Isolation and Characterisation of the 5' Region of the Bovine Lactoferrin Gene

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Dedication

This thesis is dedicated to the memory of my grandfathers,

Claud Charles Brown and John Morris Bain.

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Abstract

Lactoferrin is an iron-binding protein found in a variety of mammalian secretions and polymorphonuclear leucocytes. Many biological roles have been proposed for lactoferrin however despite extensive research, the precise function(s) of this protein remain unclear. Lactoferrin appears to be regulated in a highly specific manner, dependent upon the developmental stage, the tissue and the species being investigated. RNA analyses have suggested that transcriptional regulation may be a significant factor controlling the expression of bovine lactoferrin within the mammary gland. A detailed knowledge of the molecular mechanisms and factors involved in the transcriptional regulation of the bovine lactoferrin gene will provide insights into the biological role of this protein.

It was with this primary aim in mind that an unamplified bovine genomic library was screened for the presence of bovine lactoferrin 5' regulatory sequences. This resulted in the isolation of a clone which was characterised by detailed restriction mapping and found to contain sequences complementarity to bovine lactoferrin exon I and exon II sequences. Analysis of this clone indicated that is contained ~10 kb of DNA 5' to the transcriptional start site of bovine lactoferrin. A ~2.85 kb fragment which hybridised to an exon I probe was isolated and subjected to dideoxy sequencing. ~2.5 kb of DNA 5' to the lactoferrin coding sequence was identified within this fragment. A computer sequence homology search indicated that several putative consensus binding sites for transcription factors may be located within this DNA sequence. Fragments prepared from this subclone by PCR were subcloned into reporter gene expression constructs. The transcriptional activity of one of these constructs was investigated using COS cells and transient reporter gene expression studies. This construct, containing 565 bp of 5' bovine lactoferrin sequence, exhibited promoter activity.

The successful isolation of this promoter will enable further investigations directed towards elucidating the transcriptional regulation of the bovine lactoferrin gene to be performed.

Abbreviations

amp	ampicillin
AP-1	Activator Protein 1
AP-2	Activator Protein 2
bLf	Bovine lactoferrin
bp	base pair
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyl transferase
CMV	cytomegalovirus
COUP-TF	Chicken Ovalbumin Upstream Promoter-Transcription Factor
Da	Dalton
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethyl sulphoxide
DNase	deoxyribonuclease
BRL	Bethesda research laboratories
dTTP	deoxythymine triphosphate
EEO	electroendosomosis
EGF	Epidermal Growth Factor
ELISA	enzyme-linked immunoabsorbant assay
ERE	Estrogen response element
FBS	Fetal Bovine Serum
GCG	Genetics Computing Group
GF/A	glass fibre/A
hGH	human growth hormone
HTf	human transferrin
IPTG	isopropyl β-D-thiogalactopyranoside
IRE	iron-responsive element
kb	kilobase
kDa	kilo Dalton
λ	Bacteriophage lambda
NF-Il 6	Nuclear factor-interleukin 6
nt	nucleotide
Oct-1	Octamer transcription factor 1

PBS	Phosphate buffered saline
PEG 8000	polyethylene glycol 8000
pfu	plaque forming units
RES	reticuloendothelial system
RNase	ribonuclease
SDS	sodium dodecylsulphate
SSC	standard saline citrate
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	tris (10 mM) EDTA (1mM) pH 8.0
UTR	untranslated region
UV	ultraviolet
XGal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

List of Tables

List of Figures

Page

Table of Contents

Chapter One: Introduction

Lactoferrin, a member of the transferrin family, is an iron-binding protein found in a variety of mammalian secretions and polymorphonuclear leucocytes. Many of the physiological functions of lactoferrin are thought to be related to the ability of lactoferrin to reversibly bind two ferric ions. As no precise role for lactoferrin has been established, an understanding of the mechanisms controlling expression of this protein may provide further insight into the biological significance of lactoferrin.

Iron is a essential element for almost all living organisms. It participates in a variety of biochemical processes including oxygen and electron transport, bacterial nitrogen fixation and DNA synthesis (Aisen & Listowsky, 1980). In aqueous solution, iron can exist in two oxidation states; the ferric Fe(III) and the ferrous Fe(II) forms. Under physiological conditions the ferric form is more thermodynamically stable. However this form of iron is practically insoluble at this pH and readily hydrolyses and polymerises to form potentially toxic ferric hydroxide and oxyhydroxide polymers which render iron biologically inaccessible to the cell (Octave *et al.*, 1983). Consequently, the concentration of uncomplexed ferric ions cannot exceed ~10^{-17}M in neutral solutions. Accordingly, nature has developed specialised proteins which chelate, transport or store iron and maintain the ferric iron in a bio-available non-toxic form. The iron-binding proteins of vertebrates can be classified into three groups: (i) the iron-containing proteins with catalytic or carrier functions, (ii) the proteins of iron-storage and (iii) the iron-transport proteins (Smith *et al.*, 1983).

In normal humans ~71% of the total iron is complexed to iron-containing proteins with catalytic or carrier functions. Characteristic of this class of proteins are the heme-containing cytochromes, oxygen carriers such as haemoglobin and myoglobin, and the non-heme iron-containing proteins. The iron-storage proteins such as ferritin and hemosiderin, contain ~27% of the total body's iron. Serum transferrin is the major iron-transport protein of vertebrates (Crichton, 1985) and containing ~0.1% of the total iron complement. Transferrin plays a critical role in shuttling iron in a non-toxic, bio-available form between sites of storage and cells requiring iron for essential life processes. Cellular uptake of bound iron from transferrin occurs by a well-characterised receptor-mediated endocytosis mechanism (Thorstensen & Romslo, 1990).

The transferrin family of iron-binding proteins are monomeric glycoproteins with a molecular weight of approximately 80,000 Da. Serum transferrin is the most abundant and well characterised member of the transferrin family (Aisen & Listowsky, 1980). Other members of the family include ovotransferrin, an egg white protein (Chasteen,

1983); melanotransferrin, a membrane-associated protein found in melanocytes (Rose *et al.*, 1986); and lactoferrin found in milk and a variety of other mammalian exocrine secretions (Sørensen & Sørensen, 1939; Masson *et al.*, 1966). With the exception of melanotransferrin, all members of the transferrin family reversibly bind two ferric ions. Melanotransferrin binds a single ferric ion within the N-lobe domain (Baker *et al.*, 1992). Each ferric ion occupies a specific site within the molecule and requires the concomitant binding of a carbonate anion (Schlabach & Bates, 1975).

The transferrins are a family of highly conserved proteins. Amino acid alignments have shown that there is extensive homology between different transferrins and that each transferrin has a pronounced twofold internal homology (Metz-Boutigue *et al.*, 1984). X-ray crystallography has shown the overall structural organisation of the transferrins to be bilobal, with two globular substructures or domains, each having a binding site for one iron atom (Gorinsky *et al.*, 1979; Anderson *et al.*, 1987). Comparison of the transferrin N-lobe and C-lobe halves of the polypeptide chain has shown that ~40% of the residues are identical (Metz-Boutigue *et al.*, 1984). The marked amino acid homology and structural similarities of the transferrins suggests this family of proteins has a common phylogenetic origin, possibly arising by gene duplication of a single iron-binding ~40 kDa ancestral protein (Williams, 1982).

The organisation of the genes for human transferrin (Park *et al.*, 1985; Schaeffer *et al.*, 1987), ovotransferrin (Cochet *et al.*, 1979; Jeltsch *et al.*, 1987), murine lactoferrin (Cunningham *et al.*, 1992) and bovine lactoferrin (Seyfert *et al.*, 1994) genes support the suggestion that transferrins arose by gene duplication. Each gene consists of 17 exons interrupted by 16 introns. The size of the exons is comparable between genes (Table 1) and the coding regions of the transferrin genes are all approximately 2.3 kb in length. There are considerable differences in intron sizes resulting in an overall difference in size between the ~33.5 kb serum transferrin and the ~10.5 kb ovotransferrin gene (Bowman *et al.*, 1988).

Chromosomal mapping experiments have localised the serum transferrin and lactoferrin genes to human chromosome 3 and murine chromosome 9 (LeProvost *et al.*, 1994 and references therein). The bovine lactoferrin gene has recently been mapped to chromosome 22 (Schwerin *et al.*, 1994). In cattle the serum transferrin gene and lactoferrin gene are located on different chromosomes which indicates that the chromosomal position of these genes has not been conserved throughout evolution (LeProvost *et al.*, 1994).

Table 1: Structural similarity between genes of the transferrin family

Comparison of exon sizes of the genes for human transferrin (HTf), ovotransferrin (OTf), murine lactoferrin (MLf) and bovine lactoferrin (BLf). The line between exons 8 and 9 separates the exons of the N-terminal domain and the C-terminal domain of each gene. Data compiled from Cunningham *et al.* (1992) and Seyfert *et al.* (1994).

Exon	Exon Sizes			
	HTf	OTf	MLf	BLf
1	93	119	82	82
2	173	164	161	164
3	106	109	109	109
4	177	192	183	183
5	133	136	148	148
6	56	56	56	56
7	179	170	179	179
8	178	187	175	175
9	155	155	155	155
10	94	94	91	91
11	33	36	48	48
12	156	156	156	156
13	136	145	142	142
14	65	65	68	69
15	185	185	185	184
16	190	187	190	190
17	206	221	132	225

1.1 Lactoferrin

Lactoferrin was first recognised in milk by Sørensen and Sørensen in 1939 and later isolated from milk by Johanson in 1960. It has since been identified in a wide variety of human secretions including tears, saliva, bronchial mucous, gastric fluids, seminal fluid and cervical mucous (Lönnerdal *et al.*, 1976; Masson *et al.*, 1966) and is also a prominent component of the secondary granules of the neutrophilic leucocytes (Baggiolini *et al.*, 1970). Human lactoferrin has also been identified in some breast carcinomas (Campbell *et al., 1992*). Campbell *et al.* (1992) reported that the tissue distribution of lactoferrin varied considerably between species. Inoue and co-workers (1993) demonstrated by immunohistochemical techniques that bovine lactoferrin is located mainly at the gateways of the digestive, respiratory and reproductive systems in cattle. In contrast to human lactoferrin, bovine lactoferrin was not detected in either the gastrointestinal tract or in the pancreas. Lactoferrin has also been identified as the major estrogen inducible uterine protein in mice (Pentecost & Teng, 1987).

Moguilevsky and co-workers (1985) demonstrated that the lactoferrin in human milk and in neutrophils was immunologically identical. Subsequent amino acid and cDNA sequence data has confirmed this identity. Comparison of human lactoferrin cDNA sequence derived from mammary gland tissue and neutrophils has revealed only a few minor differences (Rey *et al.*, 1990; Rado *et al.*, 1987; Stowell *et al.*, 1991). These proteins may however differ in their glycan content (Derisbourg *et al.*, 1990). The

significance of these differences is unknown and may simply represent minor allelic differences.

The concentration of lactoferrin in milk varies considerably, depending upon the species and the lactational state of the mammary gland being investigated. Human milk contains considerably higher levels of lactoferrin than bovine milk. Despite variations in the absolute levels of lactoferrin, both human and bovine milk exhibit a similar pattern of lactoferrin expression. High levels of lactoferrin expression are found in colostrum (human <6 mg/ml, bovine 1-5 mg/ml), falling to lower levels in later lactation (human 1-2 mg/ml, bovine ~0.1 mg/ml) (Smith & Schanbacher, 1977; Sánchez *et al.*, 1988). Sánchez and co-workers (1992b) reported that the capacity of the sheep mammary gland to synthesise lactoferrin decreased markedly in the first 24 hours of lactation. During involution of the bovine mammary gland, there is a dramatic increase in the amount of lactoferrin found in the dry secretion of the gland with levels up to 50-100 mg/ml being reported (Welty *et al.*, 1976). Campbell *et al.* (1992) demonstrated lactoferrin was synthesised by human ductal cells in non-lactating breasts. Hurley and Rejman (1993) reported that bovine lactoferrin concentrations increased within mammary secretions until day 17 of involution. This was proposed to be the result of synthesis of lactoferrin by alveolar epithelial cells within the involuting gland. The pattern of lactoferrin expression sharply contrasts that of other milk proteins in bovine milk which have the highest concentrations during lactation and lowest during development and involution (Schanbacher *et al.*, 1993). This suggests that the regulation of expression of bovine lactoferrin is the opposite of other milk proteins. Furthermore, even during normal lactation the levels of lactoferrin can be dramatically increased in response to mammary gland infections (mastitis). These elevated levels (>1 mg/ml) can exist for prolonged periods (20-30 days) (Harmon *et al.*, 1976). Acute mastitis causes tissue damage due to inflammation and non-specific defence mechanisms such as oxidation and lipid peroxidation. Harmon *et al.* (1976) suggested that the increased concentration of lactoferrin resulted from increased synthesis and secretion of lactoferrin by the mammary gland rather than from an influx of neutrophils, containing lactoferrin, responding to the tissue damage.

1.2 Proposed Functions of Lactoferrin

No clear physiological role has been established for lactoferrin. Several putative functions have been proposed but much of the evidence is indirect and contradictory (Sánchez *et al.*, 1992a; Iyer & Lönnerdal, 1993). In this section, the primary roles for lactoferrin are discussed. This is not intended to be a complete review of all possible lactoferrin functions.

1.2.1 *Iron Absorption*

The higher bioavailability of iron and the higher concentration of lactoferrin in human milk compared with that in bovine milk led to the hypothesis that lactoferrin may have a role in iron absorption within breast fed infants (Lönnerdal, 1981).

In 1979, Cox and co-workers proposed that lactoferrin possessed the ability to deliver iron to specific cell surface receptors within the human gastrointestinal tract. Experiments involving lactoferrin labelled with both ^{59}Fe and ^{125}I indicated that iron was transported across the brush border while the intact lactoferrin molecule was excluded from the intestinal cells (Cox *et al.*, 1979). Subsequent studies have identified putative lactoferrin receptors in the brush border membrane vesicles from rabbits (Mazurier *et al.*, 1985), mice (Hu *et al.*, 1988), rhesus monkey (Davidson & Lönnerdal, 1988) and suckling pigs (Gislason *et al.*, 1993). Kawakami and Lönnerdal (1991) isolated a human lactoferrin receptor from solubilised human fetal intestinal brush-border membranes. Preliminary characterisation indicated that the ~114 kDa molecular weight receptor was glycosylated and composed of ~38 kDa subunits. Kawakami and Lönnerdal (1991) reported removal of the glycan chains from lactoferrin with peptide-N^4-(N-acetyl-β-D-glucosaminyl) asparagine amidase F (PNGase F) did not inhibit binding to the receptor. This contrasts with the work of Davidson and Lönnerdal (1988) on the rhesus monkey lactoferrin receptor which implicated the importance of the fucose carbohydrate moieties for receptor recognition.

Only 1-4% of lactoferrin is iron saturated in milk (Fransson & Lönnerdal, 1980). Consequently, it is conceivable that lactoferrin might bind iron within the gastrointestinal tract, simultaneously depriving bacteria of iron, and delivering iron to specific receptors within the gut. However, there is some doubt as to whether iron from lactoferrin is bio-available. *In vitro* studies using rat and guinea pig everted duodenal sacs suggested that iron uptake is inhibited by apo-lactoferrin (De Laey *et al.*, 1968). De Vet and van Gool (1974) demonstrated a negative relationship between duodenal lactoferrin concentration and iron absorption in human adults.

For lactoferrin to act as a facilitator of iron absorption in the gut, the protein must resist proteolytic digestion and survive passage through the gastrointestinal tract. Iron-loaded human and bovine lactoferrin have been shown to be more resistant to proteolytic cleavage than the iron-free, apo-form (Spik *et al.*, 1982). Various molecular forms of lactoferrin have been detected in the stools and urine of pre-term infants fed either bovine or modified human milk (Goldman *et al.*, 1990; Goldblum *et al.*, 1989). The iron status of these protein forms was not reported. These results implied that lactoferrin was absorbed across the gastrointestinal tract within these individuals (Goldblum *et al.*, 1989). However, the iron status of these protein forms was not reported.

No conclusive data has been reported to implicate the importance of lactoferrin in iron absorption from human milk. Some studies have indicated that the bio-availability of

iron bound to lactoferrin may be no better than inorganic iron (Fairweather-Tait, 1987). The significance of this study is, however, unclear as full-term 7-day-old infants were fed standard milk formula supplemented with bovine lactoferrin. The validity of experiments involving bovine lactoferrin supplementation within a milk formula needs to be questioned since the putative human lactoferrin receptor does not to bind bovine lactoferrin (Kawakami & Lönnerdal, 1991).

Davidsson and co-workers (1994), investigating healthy full-term infants which were fed either breast milk or lactoferrin-free breast milk, reported that iron absorption was significantly lower when lactoferrin was present. These results were in agreement with earlier studies performed by De Vet and van Gool (1974). Interestingly, the youngest infant showed higher iron absorption from human milk than from lactoferrin-free breast milk. This finding supported the hypothesis of Brock (1980) who proposed that the biological role of lactoferrin may change from early to late infancy.

Given the discrepancy between the suggestion that lactoferrin can enhance or inhibit iron absorption in infants, it appears critical to establish a few key parameters. Firstly, the age of the infant is likely to influence the amount of lactoferrin cleaved by proteolytic digestion within the gut. Secondly, the source of the lactoferrin used in supplementation experiments is critical in determining whether it is likely to interact with putative lactoferrin receptors within the brush border membrane. Thirdly, addition of iron may influence the binding of the molecule to the receptor since mono- or diferric-lactoferrin is known to have a higher affinity for the receptor than the apo-form (Davidson & Lönnerdal, 1989). Finally, the choice of model system investigated should be related to the levels of lactoferrin present within the milk of that species. The relevance of studies performed in rats is uncertain since rat milk contains very low levels of lactoferrin and high levels of transferrin (Kawakami *et al.*, 1988; Schanbacher *et al.*, 1993 and references therein).

The precise role of lactoferrin in iron absorption still remains unclear. Lactoferrin binding proteins have been identified within the brush border membranes of several species but there has been no direct evidence that these membrane proteins actually mediate uptake or transport of lactoferrin-bound iron to mucosal cells. Furthermore, no role has been proposed for the absorbed iron once it is taken up by enterocytes. In summary, lactoferrin may act as a regulator rather than an enhancer of iron absorption in infants (Brock, 1980).

1.2.2 *Antimicrobial*

Lactoferrin is secreted in the apo- or iron-free form. Based upon the iron sequestering properties of lactoferrin, it has been proposed that lactoferrin may possess a non-specific antimicrobial activity (Bullen *et al.*, 1972). Several investigators have

reported that patients with deficiencies in neutrophil lactoferrin are prone to recurrent infections (Breton-Gorius *et al.*, 1980; Boxer *et al.*, 1982). Both *in vivo* and *in vitro* studies indicate that lactoferrin has antibacterial activity (Ellison *et al.*, 1988 and references therein). This effect is reported to be blocked if lactoferrin is saturated with iron (Reiter, 1983; Spik *et al.*, 1978; Bishop *et al.*, 1976; Nonnecke & Smith, 1984).

The amount of bovine lactoferrin increases significantly during involution and periods of mammary infections (Welty *et al.*, 1976; Bishop *et al.*, 1976). Bovine lactoferrin has been shown to inhibit the growth of eight strains of coliform bacteria associated with bovine mastitis (Bishop *et al.*, 1976). This growth-inhibiting effect has been attributed to the iron-chelating ability of lactoferrin. Repeating these experiments using iron saturated lactoferrin or exogenous iron and apolactoferrin abolished the growth-inhibition (Bishop *et al.*, 1976). Growth inhibition was reversed by citrate, which appears to make protein-bound iron available to micro-organisms (Reiter *et al.*, 1975). The amount of citrate and lactoferrin in milk vary dramatically during involution and lactation. It is thought that the molar ratio of citrate to lactoferrin may be important in determining whether lactoferrin will exert a protective role in the mammary gland (Smith and Schanbacher, 1977).

Spik *et al.* (1978) proposed from *in vitro* studies, that rather than being solely due to lactoferrin, the observed bacteriostatic effect was the result of a synergistic interaction between lactoferrin and secretory immunoglobulin A. Neither component alone was able to provide the observed inhibition. Arnold *et al.* (1977) demonstrated that human lactoferrin could exert a bacteriocidal effect which was abolished by saturating lactoferrin with iron. Other studies have indicated that lactoferrin can damage the outer membrane of enteric gram-negative bacteria (Ellison *et al.*, 1988; Ellison & Giehl, 1991). Lactoferrin was reported to cause the release of lipopolysaccharide (LPS) molecules from the bacterial outer membrane, increasing the permeability of the membrane, and sensitising the bacteria to antibiotics, and enhancing the effect of lysozyme. Ellison and Giehl (1991) found lactoferrin and lysozyme together produced a bactericidal effect which was dose-dependent, blocked by iron saturation of lactoferrin, and inhibited by high calcium levels. Both of these proteins co-exist in milk, mucosal secretions and neutrophils and are actively secreted in response to inflammation (Ellison & Giehl, 1991 and references therein).

Many pathogenic bacterial species have been reported to utilise lactoferrin as an iron source (Mickelsen *et al.*, 1982; Peterson & Alderete, 1984; Schryvers, 1989; Naidu *et al.*, 1991; Ascencio *et al.*, 1992; Wilson *et al.*, 1994; Campagnari *et al.*, 1994). A single ~105 kDa outer membrane protein of *Neisseria meningitidis* bacterium has been identified as possessing human lactoferrin binding, independent of the level of iron

saturation (Yu & Schryvers, 1993; Schryvers & Morris, 1988). Both the N-lobe and C-lobe of lactoferrin participated in the binding interaction with bacterial lactoferrin receptors (Yu & Schryvers, 1993). Pettersson *et al.* (1994) identified the lactoferrin receptor of *Neissera meningitidis* as the iroA gene product.

Gadó and co-workers (1991) established a correlation between human lactoferrin binding and colicin susceptibility in *E. coli*. OmpF in the bacterial outer membrane has been identified as a specific receptor for colicins and lactoferrin (Erdei *et al.*, 1994). Porins are associated with LPS molecules in the bacterial membrane. Gadó *et al.* (1991) suggested the gradual degradation of LPS molecules exposed OmpF to lactoferrin and colicin, facilitating binding and antibacterial activity. Erdei and co-workers (1994) reported that neither the degree of iron saturation nor the species of origin of lactoferrin affected the interaction of lactoferrin with OmpF. This contrasts with earlier work which had demonstrated that both the antibacterial effect and the expression of bacterial lactoferrin receptors to be iron-sensitive.

The functional and structural relationship between OmpF or IroA in different pathogenic bacteria is unknown. It is possible that both of these outer membrane proteins are porins differing in their utilisation of lactoferrin-bound iron. *E. coli* secrete siderophores to acquire iron from the environment. *Neisseria* do not produce siderophores and are proposed to utilise iron bound to lactoferrin as a nutritional source. Both *E. coli* and *Neisseria* appear to be able to bind lactoferrin, however, the nature or function of this interaction appears to be different for both of these pathogens. This may reflect the different nutritional requirements of these bacteria and demonstrate that the role of lactoferrin interacting with bacteria is dependent upon the species and/or other as yet undetermined factors.

A putative bactericidal domain has recently been isolated and characterised in bovine lactoferrin (Bellamy *et al.*, 1992). This peptide, lactoferricin, is reported to be released by hydrolysis of orally ingested lactoferrin or hydrolysis of lactoferrin within the phagolysosome of polymorphonuclear leucocytes. Lactoferricin B, derived from bovine lactoferrin, has been reported to alter the structure of gram negative bacterial outer membranes by causing the release of LPS molecules (Yamauchi *et al.*, 1993; Bellamy *et al.*, 1993). For the significance of this putative peptide to be determined, it will be critical to establish whether lactoferricin is generated *in vivo*.

1.2.3 *Role as Growth Promoter*

Human lactoferrin in cell culture has been reported to be effective at promoting the proliferation of human lymphocytic cells (Hashizume *et al.*, 1983). Small changes in

lactoferrin concentration were found to dramatically affect the growth rates of human lymphocytic cells, implying that the level of lactoferrin regulated the level of cell proliferation (Hashizume *et al.*, 1983). Human lactoferrin has also been demonstrated to stimulate thymidine incorporation into DNA of rat crypt enterocytes (Nichols *et al.*, 1987). Incorporation was found to occur independently of iron-binding (Nichols *et al.*, 1990). As most of the lactoferrin in human milk is in the apo-form, this suggests that lactoferrin may aid the maturation of the intestine in the newborn. The possibility that lactoferrin acts as a growth factor is supported by the presence of high concentrations of lactoferrin in some tumours (Tuccari *et al.*, 1989). The mechanism by which lactoferrin exerts this mitogenic effect is unknown.

Mazurier *et al.* (1989) reported that lymphocytes stimulated by phytohemagglutinin expressed a specific receptor for human lactoferrin. This growth-stimulating activity was dependent upon iron saturation and occurred at lactoferrin concentrations comparable to physiological human serum lactoferrin concentrations. Resting peripheral blood lymphocytes were found to be devoid of surface or intracellular receptors for human lactoferrin, suggesting that the receptors were only expressed following a mitogenic stimulus (Mazurier *et al.*, 1989). The N-terminal domain I of human lactoferrin has been identified as the region likely to interact with the lymphocyte receptor (Rochard *et al.*, 1989). Sun *et al.* (1991) proposed that diferric lactoferrin could participate in redox reactions at the plasma membrane of K562 and HeLa cells leading to an activation of a Na^+/H^+ antiport and NADH oxidase. This may represent a possible mechanism for growth stimulation by lactoferrin.

1.2.4 *Anti-oxidant*

Oxygen, although essential for the life of aerobes, can also be highly toxic to cells due to lipid peroxidation and the generation of hydrogen peroxide (H_2O_2) and superoxide free radicals (O_2^-). When stimulated, polymorphonuclear leukocytes (neutrophils) phagocytose bacteria. Oxygen from the surrounding medium is also taken up in this process and is converted to the superoxide anion and hydrogen peroxide. Hydroxyl radicals capable of attacking and destroying almost all known biomolecules are generated by hydrogen peroxide and superoxide free radicals reacting together in the presence of activated iron. Both lipid peroxidation and hydroxyl radical formation require the presence of iron to act as a catalyst (Gutteridge *et al.*, 1981).

Lactoferrin has been identified within the secondary granules of neutrophils in the apo-form and is released into the surrounding medium in a partially iron-saturated form upon activation of the neutrophil (Masson *et al.*, 1969; Bullen & Armstrong, 1979). Debate exists as to whether lactoferrin acts as an oxidant or anti-oxidant when released by neutrophils. For lactoferrin to have an anti-oxidant role, it must bind iron and inhibit the formation of hydroxyl radicals and thus promote bacterial survival. If, however,

lactoferrin released iron into the surrounding medium, radical formation would be promoted leading to the destruction of bacterial cell membranes by lipid peroxidation followed by bacterial cell death. Ambruso and Johnston (1981) reported neutrophil lactoferrin could efficiently provide iron to the oxygen radical-generating system. This observation was supported by Bannister *et al.* (1982) who concluded that lactoferrin was indeed involved in hydroxyl radical formation. In contrast, it has also been reported that lactoferrin bound iron is a poor catalyst of oxygen radical production and lipid peroxidation and thus inhibited both of these processes (Gutteridge *et al.*, 1983; Winterbourn, 1983; Baldwin *et al.*, 1984). The reason for this discordance could lie in the failure of Ambruso & Johnston (1981) and Bannister *et al.* (1982) to exclude extraneous iron. Extra iron within the system abolishes the inhibition of lipid peroxidation (Gutteridge *et al.*, 1981). Furthermore, Ambruso and Johnston (1981) and Bannister *et al.* (1982) reported that lactoferrin was a good catalyst of oxygen radical production in the presence of EDTA and NTA, both chelating agents which affect the properties of lactoferrin (Winterbourn, 1983). Support for lactoferrin acting as an anti-oxidant was provided by Monteiro and Winterbourn (1988) who found that the superoxide anion caused rapid and efficient iron transfer from ferritin to apolactoferrin and apotransferrin. Lactoferrin within neutrophils is predominantly in the apo- or iron-free form. Therefore, lactoferrin would have to bind iron before it could promote oxidation within cells (Bullen & Armstrong, 1979).

From the current literature, it appears that lactoferrin is more likely to have a role in extracellular anti-oxidant defence than as an oxidant promoter.

1.2.5 *Inflammation*

Lactoferrin contained within the secondary granules of neutrophils is thought to play a role in inflammation which is a basic response of the body to injury of tissues caused by physical, chemical and infective agents. This response is a basic protective cascade of events against invading pathogens involving chemotaxis to the site of inflammatory infection, target recognition, ingestion via phagocytosis, killing and degradation of infective agents. The cytokine tumour necrosis factor (TNF) is a potent chemotaxin of neutrophils and has been shown to increase neutrophil responses to inflammation. TNF is also a direct stimulus of degranulation of secondary neutrophil granules and the production of toxic oxygen radicals (Crouch *et al.*, 1992).

A consistent finding in inflammation is a reduction in serum iron levels associated with an increase in serum ferritin (Gordeuk *et al.*, 1988 and references therein). The decrease in serum iron levels (hyposideraemia) may be a non-specific defence mechanism of the host to reduce the growth of invading micro-organisms.

Lactoferrin released from the secondary granules of neutrophils is in the iron-free or apo-form. At acidic pHs, such as those observed during inflammation, the binding

constant of ferric iron is approximately 300 times higher for lactoferrin than transferrin (Aisen and Leibman, 1972). Van Snick *et al.* (1974) proposed that lactoferrin released from degranulating neutrophils caused localised anaemia (hyposideraemia) by removing iron from transferrin and being selectively taken up by the reticuloendothelial system (RES) causing accumulation of iron within ferritin. The presence of a specific lactoferrin receptor on monocytes was confirmed by Birgens *et al.* (1983).

Subsequent studies have questioned this theory. The iron uptake by macrophages from lactoferrin is extremely slow (Oria *et al.*, 1988). No recycling of lactoferrin occurs. Once bound to monocytes, lactoferrin cannot subsequently rebind to these cells (Birgens & Kristensen, 1990). Derisbourg *et al.* (1990) have demonstrated that neutrophil-derived lactoferrin lacks the terminal fucose residues in the glycan chains which have been implicated as a requirement for binding to macrophages. In addition, Gordeuk *et al.* (1988) have found that interleukin-1 (IL-1) administered to mice induces hypoferraemia even in the presence of neutropenia (deficiency of granulocytes), suggesting lactoferrin is unlikely to play a significant role in hypoferremia of inflammation. Crouch and co-workers (1992) reported that lactoferrin inhibited the release and not the action of IL-1, Interleukin-2 (IL-2) and TNF and proposed that this may provide an inhibitory feedback mechanism to prevent excessive neutrophil recruitment and activation at an inflammatory site. Machnicki and co-workers (1993) demonstrated treatment of mice with bovine lactoferrin prior to infection with *E. coli* induced an increase in IL-6, an inhibitor of TNF-α production, in serum.

In summary, it appears that the role of lactoferrin in inflammation may be to modulate the levels of key cytokines. The mechanism by which this occurs remains to be elucidated.

1.2.6 *Iron Scavenger*

Human plasma contains approximately 0.2-1.5 μg of lactoferrin per millilitre (Regoeczi *et al.*, 1985 and references therein). Circulating lactoferrin is thought to be derived from neutrophils which release apo-lactoferrin from their secondary granules (Baggiolini *et al.*, 1970). Lactoferrin differs from transferrin by possessing one or two fucosyl residues in each of its glycans. As a consequence of these moieties, lactoferrin resembles partially desialylated transferrin. *In vivo* human asialotransferrin is taken up by the liver and passes through repeated endocytosis and exocytosis cycles. Recently, it has been demonstrated that bovine and human lactoferrin interfere with the uptake of iron from transferrin and asialotransferrin by rat liver (Hu *et al.*, 1993). Lactoferrin is known to be rapidly cleared from circulation by the liver following intravenous injection (Prieels *et al.*, 1978; Regoeczi *et al.*, 1985). Regoeczi *et al.* (1985) proposed that the number of lactoferrin receptors required to account for the clearance rates observed in rats would preclude the presence of specific receptors. Once taken up by the liver, subcellular

lactoferrin is located within the endosome and the lysosome (Hu *et al.*, 1993). The dual location of lactoferrin was suggested to implicate lactoferrin participating in repeated plasmalemma-to-lysosome shuttles. McAbee *et al.* (1993) proposed that lactoferrin may function as an iron scavenger within the hepatocyte endocytic pathway.

Lactoferrin has been shown to bind to liver parenchymal cells (McAbee and Esbensen, 1991). The binding of lactoferrin to purified rat liver plasma membranes has been reported to involve electrostatic interactions leading to specific binding. Ziere *et al.* (1993) characterised the binding as low affinity (K_d=10 μM) but involving high capacity (20 x10^6 binding sites/cell). Hepatic heparan sulphate proteoglycan (HSPG) molecules present within the endosome and lysosome membranes possess an affinity for lactoferrin (Hu *et al.*, 1993). The mechanism by which this occurs is unclear although it appears that two features may be essential for the interaction of lactoferrin with HSPG and its interference with hepatic iron uptake from rat transferrin and rat asialotransferrin. Studies by Ziere *et al.* (1992, 1993) have led to the proposal that a cluster of arginine residues in the N-terminal domain may confer binding of lactoferrin to parenchymal cells. The proteins isoelectric point (~8.7) may also be an essential feature for the interaction of lactoferrin with HSPG (Hu *et al.*, 1993; Moguilevsky *et al.*, 1985 and references therein). Debanne *et al.* (1982) found other proteins with a high pI were able to inhibit human lactoferrin binding to rat liver plasma membranes.

Hu *et al.* (1993) proposed the binding of lactoferrin to HSPG prevented transferrin binding to the proteoglycans. The inability of transferrin to bind to the hepatic plasma membrane would prevent uptake of transferrin and therefore maintain iron bound to transferrin within circulation.

Much of the above evidence is indirect and speculative. These findings however imply lactoferrin may act as an iron scavenger by interacting with HSPG molecules in the hepatocyte endocytic pathway.

1.2.7 *Lactoferrin as a regulator of myelopoiesis*

The production of granulocytes and macrophages is a dynamic process, dependent upon cell-derived molecules which control the proliferation and differentiation of granulocyte-macrophage progenitor cells. *In vivo* these interactions tightly regulate granulocyte and monocyte numbers (Broxmeyer *et al.*, 1980).

Lactoferrin has been implicated as a potential negative feedback regulator of myelopoiesis on the basis of its' ability, in the iron-saturated form, to suppress the production of granulocytes and macrophages in intact mice and *in vitro* (Broxmeyer *et al.*, 1978; Broxmeyer *et al.*, 1980; Gentile & Broxmeyer *et al.*, 1983). Broxmeyer and co-workers (1987) reported that lactoferrin bound to specific receptors on monocytes and macrophages and suppressed the production of granulocyte-macrophage colony stimulatory factors (GM-CSF) or monokines, which were necessary for stimulating the

proliferation and differentiation of myeloid progenitor cells. This was proposed by Zucali *et al.* (1989) to occur by lactoferrin inhibiting the synthesis and release of interleukin 1 (IL-1), a cytokine known to stimulate the production of GM-CSF. Lactoferrin has also been reported to inhibit the production of TNF-α, an activator of IL-1, and to increase the synthesis and secretion of interleukin-6 (IL-6), an inhibitor of TNF-α (Crouch *et al.*, 1992; Machnicki *et al.*, 1993).

All these experiments have utilised iron-saturated lactoferrin despite lactoferrin being released in the apo- or iron-free form from neutrophils. Broxmeyer *et al.* (1980) proposed that apo-lactoferrin bound iron from the serum, inducing a conformational change which facilitated the binding of iron-saturated lactoferrin to monocytes and the suppression of GM-CSF production. This scenario is somewhat controversial as other investigators have failed to demonstrate an inhibitory effect of lactoferrin on myelopoiesis *in vitro* (Winton *et al.*, 1981). In addition, the levels of free iron within serum are extremely low and at physiological pH, transferrin and lactoferrin have similar binding affinities for ferric iron (van Snick *et al.*, 1974; Mazurier & Spik, 1980). Further investigations are clearly required to establish whether lactoferrin has a regulatory effect on myelopoiesis.

1.3 Regulation of Transferrin Gene Expression

The transferrin gene is expressed and regulated by a complex process involving multiple regulatory elements interacting with the DNA in an organ- and gene-specific manner (McKnight *et al.*, 1983; Bowman *et al.*, 1988).

The principal site of transferrin synthesis is the liver, although other sites of synthesis such as testis, brain, spleen and kidney have been identified (Skinner & Griswold, 1980; Idzerda *et al.*, 1986).

1.3.1 *Modulating Factors*

The expression of transferrin is modulated principally by iron and hormones. The avian transferrin gene is active in both the liver and oviduct. Liver transferrin and ovotransferrin (egg white) have been found to be products of the same gene, differing only in their respective carbohydrate moieties (Williams, 1962; Thibodeau *et al.*, 1978). The expression of the avian transferrin gene is however modulated quite differently in the two tissues. The liver and oviduct are both steroid-responsive tissues, however the expression of ovotransferrin has been shown to be more sensitive to steroid hormones than serum transferrin expression (Lee *et al.*, 1978). Both estrogen and progesterone induce a marked stimulation of ovotransferrin synthesis. The stimulation of transferrin synthesis by estrogen is less pronounced while progesterone has no effect (Lee *et al.*, 1978). Tissue iron levels appear to be important in regulating the expression of the

transferrin gene within the liver, but not in the oviduct (McKnight *et al.*, 1980b). During periods of iron deficiency, transferrin gene expression is induced specifically within the liver by modulation of mRNA levels (Idzerda *et al.*, 1986; McKnight *et al.*, 1980a). Iron deficiency combined with estrogen supplementation *in vitro* has been shown to result in an additive response (McKnight *et al.*, 1980b). This suggests that iron deficiency and estrogen interact with the liver transferrin gene through separate regulatory mechanisms (McKnight *et al.*, 1980b).

In humans, iron overload causes a decrease in transferrin expression while an iron deficiency results in an increase in transferrin expression (Cox & Adrian, 1993 and references therein). This iron-responsive effect is thought to be mediated by a putative iron-responsive element (IRE) which is a stem-loop structure within the first 46 nucleotides of the human transferrin 5'-UTR (Cox & Adrian, 1993). *In vitro* binding assays performed by Cox and Adrian (1993) demonstrated specific binding of liver cytoplasmic proteins to this region of the transferrin mRNA. Decreased binding was observed in experiments using cytoplasmic extracts prepared from iron-treated mice. Cox and Adrian (1993) suggested that an increase in binding of cytoplasmic factors to this region of the transferrin gene resulted in an increase in translation of the human transferrin mRNA. Competition binding assays demonstrated that the HTf 5'-UTR binding protein(s) also exhibited an ability to bind to the ferritin IRE (Cox and Adrian, 1993).

The *in vivo* response of human transferrin to iron was investigated in transgenic mice carrying chimeric genes composed of human transferrin (HTf) 5'-flanking sequences fused to the reporter gene chloramphenicol acetyl transferase (CAT). Liver-specific expression of the protein product of the HTf-CAT transgene was found to be suppressed following intraperitoneal injections of iron, however the liver HTf-CAT mRNA levels were unaffected by iron treatment. This suggested that the iron regulation of human transferrin expression was post-transcriptional.

Further characterisation of the transferrin iron responsive elements will be necessary to identify the cytoplasmic binding proteins involved. The findings of Cox and Adrian (1993) imply that a common cytoplasmic binding factor may be involved in the iron modulation of transferrin, ferritin and the transferrin receptor.

1.3.2 *Transcription Regulatory Elements*

Transcription is one of the central control points in the regulation of eukaryotic gene expression. The human transferrin gene is expressed at a high level in the liver and at a low level in the testis (Skinner & Griswold, 1980). In order to investigate the control mechanisms for transferrin gene expression, the 5' untranslated region of the transferrin gene has been isolated and investigated extensively.

Transient and stable expression of deletion mutants of the 5' region of the human transferrin gene using hepatoma cells, identified four distinct functional regions which have been implicated in the regulation of transcription of the transferrin gene (Schaeffer *et al.*, 1989; Brunel *et al.*, 1988). These regions included: 1) a proximal promoter region; 2) a distal promoter region; 3) a negative acting region and 4) an upstream enhancer element.

The proximal promoter region has been shown to be essential for transferrin gene transcription and contains a putative liver specific element thought to direct expression of the transferrin gene specifically to the liver (Brunel *et al.*, 1988). *In vitro* binding assays have identified five protein binding sites in the first 520 nucleotides of the 5' region of the transferrin gene. Two of these sites are localised in the proximal or tissue-specific promoter region. These are proximal region I (PRI) covering nucleotides -76 to -51 and proximal region II (PRII) which includes nucleotides -103 to -83.

Comparison of transient expression experiments suggests that cell specific transcription of the transferrin gene is governed in different tissues by a distinct combination of transcription factors. Interestingly, analysis of transcription of the human transferrin gene in hepatoma and Sertoli cells has established that the different cell-specific protein factors interact with identical cis-acting proximal promoter elements (Schaeffer *et al.*, 1993). Schaeffer and co-workers (1993) identified some of the trans-factors binding to the transferrin promoter in hepatoma and Sertoli cells (Table 2).

Data obtained indicated that at least three different PRI-binding proteins were interacting at the proximal region I. Neither CAAT enhancer binding protein (C/EBP) nor hepatic nuclear factor-4 (HNF-4) proteins are present in testis. Extension of these studies has suggested that the role of chicken ovalbumin upstream promoter-transcription factor (COUP-TF) varies between cell types. COUP-TF is a weak positive activator of transferrin expression in the liver hepatoma cells, while in testis Sertoli cells it acts as a strong negative modulator. The precise interactions between the trans-acting proteins and the identity of all of the factors involved remains to be elucidated and characterised.

Table 2: Putative transferrin trans-acting factors
Compiled from data reported by Schaeffer and co-workers (1993).

Binding Site	Nucleotides	Liver	Sertoli
PRI	-76 to -51	HNF-4 COUP-TF	SP-A SP-D COUP-TF
PRII	-103 to -83	C/EBP-related proteins	SP-α SP-β

The negative regulatory region between nucleotides -1000 and -620 of the transferrin 5' region down-regulates transcription from the transferrin promoter and is only active in the absence of the 5' enhancer region (Schaeffer *et al.*, 1989). This enhancer element located between nucleotides -3600 to -3300 has been found to be active in hepatoma cells. Transient expression studies and *in vitro* DNA protein binding assays have indicated that the transferrin enhancer is organised into two distinct structural and functional domains, A and B. Domain A binds two protein factors, one of which is HNF-3α and has been identified as being crucial for enhancer activity (Zakin, 1992 and references therein). Domain B displays no enhancer activity by itself although it can block the activity of a downstream negative element (Boissier *et al.*, 1991). The enhancer element is not active in Sertoli cells (Guillou *et al.*, 1991).

1.3.3 *Expression of Chimeric Genes*

Transgenic mice containing truncated segments of HTf 5'-flanking region linked to the chloramphenicol acetyl transferase (CAT) reporter gene (Gorman *et al.*, 1982) were used to characterise the transferrin gene sequences that respond to cellular factors and signals *in vivo* (Adrian *et al.*, 1990). Transgenes containing HTf 5'-flanking sequences from -152 to +46 attached to CAT were found to be poorly expressed in all tissues investigated. Chimeric genes containing 622 and 1152 bp of HTf 5'-flanking regions were reported to be expressed at high levels in the brain and liver. The levels of CAT enzymatic activity were higher in the brain than the liver. This is the reverse of the endogenous transferrin gene expression. The reason for this and the minimal expression within the testis is unknown, however this may demonstrate a species-specific factor difference between mice and humans.

Both of these chimeric genes were found to be iron responsive. Administration of iron produced a significant decrease in CAT enzymatic activity in the liver, a result consistent with the *in vivo* situation in humans following iron overload. 5' deletion analysis indicated that the sequence between -622 bp and +46 bp was adequate to produce this iron response (Adrian *et al.*, 1990).

Based upon the evolution and structural similarity of transferrin and lactoferrin, the mechanism and the factors influencing transferrin gene regulation may provide an insight into similar types of control mechanisms which may influence the expression of lactoferrin.

1.4 Regulation of Lactoferrin Expression

1.4.1 *Mouse Lactoferrin*

Early reproductive events such as fertilisation and embryo implantation occur in the uterine luminal fluid (ULF) of mammals. The expression of many of the proteins present within the ULF are modulated by steroid hormones. In 1986, Teng *et al.* isolated an estrogen-inducible protein from immature mouse uterine secretions. Further characterisation identified this protein as lactoferrin. Estrogen treatment was reported to result in at least a 300 fold increase in the level of lactoferrin mRNA within immature mouse uteri. Neither testosterone nor progesterone were found to induce secretion of lactoferrin (Pentecost & Teng, 1987). Similarly, Yu and Chen (1993) reported that the production of lactoferrin mRNA and protein in the prepubertal period could be stimulated by estrogen but not testosterone in the mouse epididymis. Earlier studies by Green and Pastewka (1978) reported that lactoferrin levels increased in the presence of increasing amounts of prolactin within mammary explants from pregnant mice. Analysis of lactoferrin mRNA and protein levels in the reproductive tract of adult mice has shown expression levels vary with the stage of the estrous cycle and hormonal fluctuations (Newbold *et al.*, 1992; Walmer *et al.*, 1992; McMaster *et al.*, 1992). This confirmed that the *in vitro* stimulation of lactoferrin expression by estrogen did occur within physiological systems (Teng *et al.*, 1986; Newbold *et al.*, 1992). McMaster *et al.* (1992) reported that estrogen-stimulated lactoferrin expression was blocked by progesterone although progesterone alone exhibited minimal effect on uterine lactoferrin mRNA levels. These findings suggested that lactoferrin uterine gene expression may be regulated by a balance between estrogen and progesterone.

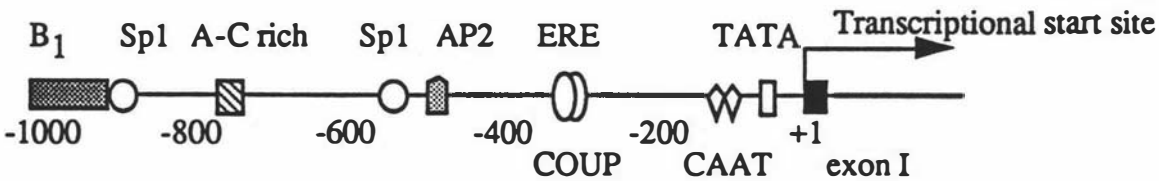
McMaster and co-workers (1992) also reported that the highest concentrations of lactoferrin in mouse uterine secretions occurred on day one and day two of pregnancy. Protein levels decreased progressively to a low level on day four of pregnancy in mice. The relative rate of uterine synthesis of lactoferrin and the abundance of neutrophils in the uterus was highest in the early implantation period when the uterus was under the influence of estrogen. The decline in lactoferrin levels during pregnancy paralleled the decline in estrogen and may also reflect the successive increase in progesterone levels as implantation and pregnancy proceeds (McMaster *et al.*, 1992). The elevated levels of lactoferrin which occured after mating were proposed to protect the mucosal surfaces from infection and inflammatory agents likely to have been introduced into the uterus during mating. In contrast, Dalton and co-workers (1994) reported lactoferrin mRNA levels did not change in the oviducts of mice during the preimplantation period. These investigators proposed that lactoferrin may influence embryonic development by preventing an inflammatory response to mating and thus shielding the developing embryo from potentially harmful reactive oxygen species. Immunostaining of uteri at the

preimplantation period indicated lactoferrin was associated with luminal and glandular epithelial cells and also within neutrophils located in the uterine stroma. Walmer *et al.* (1992) reported polymorphonuclear leukocytes also exhibit cyclic fluctuations in the uterus during the natural estrous cycle and based upon this finding proposed that lactoferrin may have an intracellular and extracellular function within the mouse uterus. Newbold *et al.* (1992) suggested lactoferrin synthesis by uterine epithelial cells may act as a 'chemoattractant' for other cells such as macrophages, eosinophils and neutrophils and thus recruit these cells to a potential site of inflammation.

Examination of the expression of the lactoferrin gene in the adult mouse mammary gland and uterus under various physiological conditions indicated that the regulation of lactoferrin expression was tissue-specific (Teng *et al.*, 1989). Estrogen regulated the synthesis and secretion of lactoferrin in a time- and dose-dependent manner in the uterus but not in the mammary gland. Indeed, lactoferrin mRNA and protein levels in the lactating mammary gland did not correlate with the observed circulating estrogen levels (Teng *et al.*, 1989).

Characterisation of the 5'-flanking region of the mouse lactoferrin gene has identified several regions of putative cis-regulatory elements (Figure 1). The elements conferring basal lactoferrin promoter activity reside within nucleotides -234 to +1 relative to the transcriptional start site.

Figure 1: Schematic representation of the putative regulatory elements in the 5'-flanking region of the mouse lactoferrin gene (Liu & Teng, 1992)

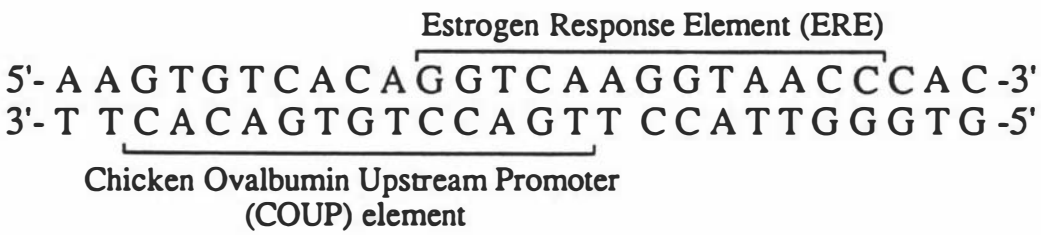


The expression of constructs containing 5' and 3' deletion mutants of the mouse lactoferrin 5' flanking region coupled to the chloramphenicol acetyl transferase (CAT) reporter gene was investigated in RL 95-2 human endometrial cells and rat C6 brain glioma cells. These studies identified a very strong positive regulatory element active in RL 95-2 cells between -1739 and -922 relative to the transcriptional start site. Expression of the same chimeric gene in C6 glioma cells was markedly reduced, indicating that expression of mouse lactoferrin is regulated in a tissue-specific manner. Transient expression of a chimeric gene containing 0.6 kb of 5' mouse lactoferrin sequences attached to the CAT gene demonstrated estrogen responsiveness in human endometrial cells in the presence of the estrogen receptor. Sequence analysis of this region revealed

an imperfect estrogen responsive element (ERE) consensus sequence overlapping with a chicken ovalbumin upstream promoter (COUP) binding element at position -349 to -329 from the transcription initiation site (Figure 2) (Liu & Teng, 1991). Mobility shift assays showed that both the estrogen receptor and the COUP-transcription factor (COUP-TF) specifically interacted with this region of the lactoferrin promoter (Liu & Teng, 1992). Band shift assays and DNase I footprinting experiments established that the COUP-TF binding element repressed the mouse lactoferrin response to estrogen stimulation. This was reported to be caused by direct competition by the COUP-TF and the estrogen receptor for a shared identical contact site within the overlapping binding regions (Liu *et al.*, 1993). Additional factors must influence the expression of lactoferrin as both the estrogen receptor and COUP-TF are present within the liver but lactoferrin is not expressed within this tissue (Pentecost & Teng, 1987).

Figure 2: Mouse lactoferrin estrogen response module

Region -351 to -316 of the mouse lactoferrin promoter showing the overlapping estrogen responsive (-341 to -329) and chicken ovalbumin upstream promoter elements (-349 to -337) (Liu *et al.*, 1993).



In vivo epidermal growth factor (EGF) can mimic estrogen stimulation of mouse lactoferrin mRNA and protein (Nelson *et al.*, 1991; Shi & Teng, 1994). EGF is present within mouse uteri (Dalton *et al.*, 1994) and estrogen enhances the expression of EGF and EGF receptor in uteri (Nelson *et al.*, 1991). Accordingly, Nelson *et al.* (1991) proposed that EGF may act as an estrogen-inducible mediator of *in vivo* expression within the mouse reproductive tract. Recently, a mitogenic-response unit has been characterised within the mouse lactoferrin promoter (Shi & Teng, 1994). A cluster of sequence elements that responded to cyclic AMP (cAMP), 12-O-tetradecanoylphorbol-13-acetate (TPA) and epidermal growth factor/recombinant transforming growth factor α (EGF/TGF-α) were located between nucleotides -80 and -40 of the mouse lactoferrin promoter (Shi & Teng, 1994). The cyclic AMP-response element (CRE) at position -52 to -40 of the mouse lactoferrin gene conferred transcriptional activation in the presence of forskolin, cAMP and TPA in transiently transfected human endometrium carcinoma RL 95-2 cells. Mobility shift assays indicated that AP-1 and CREB or related transcription factors may bind to this element. It is not yet known if the CRE in the mouse lactoferrin gene is active *in vivo*. The EGF/TGF-α response element was located within nucleotides -80 to -60. Oligonucleotides to CREB, Sp1, AP-1 and the mouse lactoferrin CRE were

unable to abolish uterine nuclear proteins binding to the EGF/TGF-α regulation site, indicating other proteins were interacting with this region. Although the EGF/TGF-α and cAMP/TPA induced regulation was conferred by two separate functional regions, mutations in either region reduced the basal transcription and transcriptional activity of the intact enhancer element. Based upon this finding, the authors proposed the proteins bound to these two elements may influence the function of the other (Shi & Teng, 1994). Further investigations are necessary to identify the mitogenic response binding protein(s) and to determine if there is a functional relationship between the identified protein(s) and the estrogen receptor.

1.4.2 *Human Lactoferrin*

Lactoferrin synthesis within developing neutrophils has been proposed to occur in a tightly regulated stage-specific manner (Rado *et al.*, 1984). Friedman *et al.* (1991) reported the rate of transcription initiation increased for lactoferrin during the early stages of terminal myeloid differentiation. This coincides with the development of secondary granules within neutrophils (Rado *et al.*, 1984). Tumour necrosis factor (TNF-α) which is produced by activated monocytes and has a central role in the inflammatory response, has been suggested as a transcriptional repressor of lactoferrin in human bone marrow (Srivastava *et al.*, 1991). In contrast to this, Campbell *et al.* (1992) reported that lactoferrin expression was more prevalent in human breast carcinomas containing TNF-α. Epidermal growth factor receptor and the estrogen receptor have also been implicated in the modulation of lactoferrin expression within human breast tumours. Campbell and co-workers (1992) reported down-regulation of lactoferrin synthesis in some forms of cancer.

Johnston *et al.* (1992) and Teng *et al.* (1992) reported the isolation and partial characterisation of the human lactoferrin promoter from placenta and HL 60 cells. Teng *et al.* (1992) identified several cis-acting elements which corresponded to sequences found in corresponding positions within the mouse lactoferrin promoter. This group also suggested that a different mechanism of estrogen action was responsible for the control of lactoferrin gene expression in mice and humans. Both mouse and human lactoferrin promoters contain an estrogen response element (ERE) which overlaps a chicken ovalbumin upstream promoter element (COUP). Teng and co-workers (1992) demonstrated the COUP-transcription factor did not compete for this binding site with the estrogen receptor in the human lactoferrin promoter. This is contradictory to the binding of these factors within the mouse lactoferrin promoter and may illustrate a species specific mechanism of regulation.

1.4.3 *Bovine Lactoferrin*

Schanbacher and co-workers (1993) demonstrated that the lactational stage-specific differences in concentrations of bovine lactoferrin resulted from changes in mRNA levels rather than a dilution effect caused by the synthesis of other milk proteins in normal lactation. Lactoferrin mRNA levels at the various developmental and lactational stages paralleled the lactoferrin protein levels in the mammary secretions. All lactoferrin mRNA levels were quite low compared to samples of early gestation (5 months) and late involution (3 and 14 days). Overall, the lactoferrin expression was found to be different to other milk proteins and highly stage specific. Schanbacher and co-workers (1993) proposed that features present within the bovine lactoferrin mRNA but absent from the human lactoferrin mRNA may contribute to the differences in lactoferrin levels in the milk of the two species. These features included a putative 5' stem-loop structure involving the AUG translational start codon and a destabilising sequence in the 3' untranslated region of the bovine lactoferrin mRNA.

Recently Seyfert and co-workers (1994) reported the characterisation of 1006 bp of bovine lactoferrin 5' sequence. A search for known conserved sequence transcriptional motifs identified a putative TATA box between nucleotides -27 to -21 (TAAAGGG), three putative CAAT binding sites (GCAAT) at nucleotides -967, -910 and -733 and three putative Sp1 recognition sites. The location of the Sp1 transcription factor regions (-196, -63 and +95 within the first exon) were very similar to potential Sp1 binding sites within the mouse lactoferrin promoter (Liu & Teng, 1991). The recognition sequences for transcription factors demonstrated to be located within human and mouse lactoferrin and serum transferrin genes such as, GATA-1, Oct-1, COUP, AP-2, estrogen response element and acute phase response element, could not be detected within the bovine lactoferrin 5' sequences. The recognition site for the mammary gland specific transcription factor (MPBF) was also absent from the isolated bovine lactoferrin promoter sequence (Schmidt-Ney *et al.*, 1991; Seyfert *et al.*, 1994). Seyfert and co-workers (1994) proposed the lack of these sequence enhancer motifs found in both human and mouse lactoferrin promoters was responsible for the relatively weak expression of bovine lactoferrin. The human lactoferrin promoter region exhibited high sequence homology to the bovine lactoferrin promoter sequence. Four regions of 30 base pairs in length or greater exhibited at least 83% homology to sequences within the bovine promoter. These were located around nucleotides -300, -377, -584 and -623 of the bovine lactoferrin gene. The significance of these regions is unknown at this stage of analysis. Further investigations are required to determine if these conserved motifs indicate conserved transcription factor binding sites and conserved regulatory mechanisms.

1.5 Justification and Aim of Project

Lactoferrin is an iron-binding protein found in a variety of cells and tissues in many mammalian species. The regulation of lactoferrin appears to be controlled in a highly specific manner, dependent upon the developmental stage, the tissue and the species being investigated. Protein levels of lactoferrin vary dramatically. Significant levels of lactoferrin are detected within polymorphonuclear leukocytes (neutrophils), mammary and uterine secretions, and lactoferrin is a constituent of most mucosal secretions and is found at lower levels in serum. Many biological roles have been proposed for lactoferrin, but despite extensive research the precise function(s) of this protein remains unclear. Lactoferrin has been reported by several researchers to be tightly regulated in a tissue-, developmental-, and species-specific manner. A detailed knowledge of the mechanisms and factors modulating the expression of lactoferrin is likely to provide insights into the functional significance of this protein.

At the outset of this project the compete cDNA sequence of bovine lactoferrin had not been determined. During the course of this work several groups have published the full length cDNA sequence. Seyfert *et al.* (1994) has also published a portion of the bovine lactoferrin promoter region. However, these investigators failed to demonstrate promoter activity.

Analysis of putative promoter regions routinely involves linking the sequences of interest to a reporter gene and introducing these genes by transfection into eukaryotic cells. The relative amount of the reporter protein synthesised is presumed to reflect the ability of the promoter to direct transcription. Three reporter genes are commonly used; chloramphenicol acetyl transferase (CAT), human growth hormone (hGH), and luciferase. The protein products of these genes are readily detected and quantitated within cell extracts.

The primary aim of this project was to isolate and characterise the 5' genomic sequences of bovine lactoferrin. The successful isolation of this DNA sequence and subsequent analysis will provide insights into the molecular mechanisms controlling the expression of the bovine lactoferrin gene.

Chapter Two: Materials and Methods

2.1 Materials

Restriction endonucleases and DNA modifying enzymes were obtained from the following companies: Life Technologies Inc., MD, USA; Promega Corporation, WI, USA; Boehringer Mannheim, West Germany; Stratagene, La Jolla, CA, USA; New England Biolabs Inc., MA, USA.

The following reagents and chemicals were purchased from Life Technologies Inc., MD, USA: Proteinase K, 1 kb DNA molecular size ladder, Random Primer Labelling System, yeast extract, bacteriological agar, penicillin, streptomycin, trysin-EDTA and DMEM.

X-gal, IPTG, low EEO type I-A agarose, ficoll, polyvinylpyrrolidone, PEG 8000, SDS, lysozyme, DNase, RNase, ampicillin, tetracycline, chloramphenicol, ethidium bromide, maltose, bovine serum albumin (Fraction V), DMSO, mineral oil and cell culture grade gelatin were purchased from Sigma Chemical Company, St. Louis, MO, USA.

Prime-a-Gene labelling system; ØX174 and λ DNA molecular size markers; Magic Miniprep DNA Purification System; Magic or Wizard Maxiprep DNA Purification System; Luciferase Reporter Vector and Assay Reagents; *Taq* DNA polymerase, Reaction Buffer and magnesium chloride were obtained from Promega Corporation, WI, USA.

The λ EMBL3 bovine genomic library was purchased from Clonetech Laboratories Inc., CA, USA. The λDASH II and the λFIX II bovine genomic libraries were obtained from Stratagene, La Jolla, CA, USA.

pCMV/hGH was a gift from Professor Floyd Schanbacher, O.A.R.D.C., Ohio State University, OH, USA.

The bovine lactoferrin cDNA was a gift from Paul Mead.

Surpon Pattanajitvillai, O.A.R.D.C., Ohio State University, OH, USA, provided p5'-350 bLf plasmid.

Radioisotopes were purchased from ICN Biomedicals Inc, New England Nuclear Research Products, Boston, MA, USA or Amersham (UK).

GeneScreen Plus was obtained from DuPont, USA. Other hybridisation filters (82 and 150 mm) nitrocellulose and nylon membranes were from Amersham, Schleicher and Schuell (West Germany) or Millipore Corporation, MA, USA.

Sequenase version 2.0 and Casein hydrolysate were from United States Biochemicals, Cleveland, OH, USA.

Oligonucleotides were manufactured by Oligos Etc. Inc., CT, USA.

dATP, dCTP, dGTP, dTTP and pCH110 were from Pharmacia, LKB Biotechnology, Uppsala, Sweden.

Epicurian Coli competent cells, *Pfu* DNA polymerase and NucTrap push columns were from Stratagene, La Jolla, CA, USA. Gelase was obtained from Epicentre Technologies, WI, USA. Dynabeads® M-280 Streptavidin were purchased from Dynal A.S., Oslo, Norway. GeneClean was obtained from BIO 101 Inc., La Jolla, CA, USA. 0.2 µm sterilisation filters were from Millipore Corporation, MA, USA. SeaPlaque and NuSieve low melting point agarose were purchased from FMC BioProducts, Rockland, ME, USA. GF/A filters and 3MM paper were from Whatmann, England.

Polaroid film was from Polaroid Corporation, Cambridge, MA, USA. X-ray film was obtained from Eastman Kodak, NY, USA or Fuji Photo Film Company Ltd., Japan. Photographic developer and fixer was purchased from Eastman Kodak, NY, USA.

Tissue culture flasks and plates were obtained from Falcon, NJ, USA.

All other chemicals and reagents were of analytical grade or similar quality.

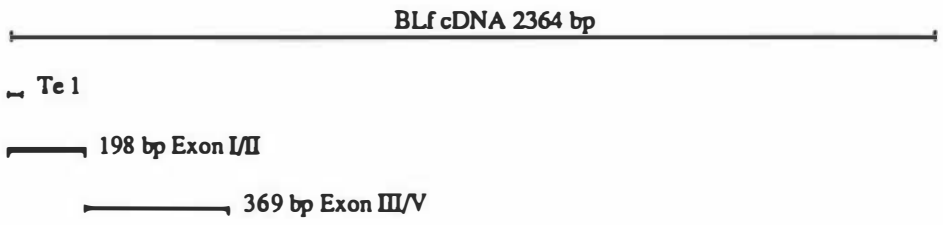
Probes Used for Screening

Probes used for the initial screening were isolated from a partial bovine lactoferrin cDNA gifted by Paul Mead. Following the isolation of the complete bovine lactoferrin cDNA, a 5' 198 bp fragment was used in the identification of 5' clones. A schematic representation of the regions of the cDNA used is shown in figure 3.

The probes used in these studies were prepared by restriction endonuclease digestion of clones containing bovine lactoferrin cDNA sequences. The 198 bp probe corresponding to exon I and exon II sequences was prepared by Hae III and Hinf I restriction endonuclease digestion of a M13 subclone, containing nucleotides 1-294 of the bovine lactoferrin cDNA sequence. The 396 bp probe was generated by cleaving a subclone, designated PM7 (Mead & Tweedie, 1990), with the restriction endonuclease Hinc II. The exon/intron boundaries of the human transferrin gene had been defined by comparison of the human transferrin cDNA and genomic sequences (Park *et al.*, 1985; Schaeffer *et al.*, 1987). Alignment of the bovine lactoferrin and human transferrin cDNA sequences (Yang *et al.*, 1984; Mead & Tweedie, 1990) enabled the putative exon regions to be defined within the bovine lactoferrin cDNA sequence. This was the basis for the assignment of exon regions contained within the probes used in these investigations.

Figure 3: Schematic representation of probes used for screening

Te1 represents a synthetic oligonucleotide



2.2 Methods

All general techniques for DNA manipulation such as ethanol precipitation, cloning of DNA fragments and phenol/chloroform extraction of DNA were performed according to standard protocols (Sambrook *et al.*, 1989a; Ausubel *et al.*, 1989). The general precautions for handling DNA described in the above texts were also observed. The strains of *E. coli* used in this study and their genotypes are outlined in table 3.

Table 3: Bacterial Strains of *Escherichia coli* K12 used in this study

Strain	Genotype
XL-1 Blue MRA(P2)	*Δ(mcrA)183, Δ(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, gryA96, relA1, lac* (P2 lysogen)
XL-1 Blue MRA	*Δ(mcrA) 183, Δ(mcrCB-hsdSMR-mrr) 173, endA1, supE44, thi-1, gryA96, relA1, lac*
C600hfl	*e14⁻(mcrA), supE44, thi-1, thr-1, leuB6, lacY1, tonA21*
SRB(P2)	*e14⁻(mcrA),Δ(mcrCB-hsdSMR-mrr) 171, sbcC, recJ, uvrC, umuC*::Tn*5* (kan^r), *supE44, gyrA96, relA1, thi-1, endA1* [F' *proAB lacI^{q}ZΔM15*], *lac* (P2 lysogen)
XL-1 Blue	*supE44, hsdR17, relA1, endA1, gyrA96, thi-1, relA1, lac⁻*, [F' *proAB⁺, lacI^q, lacZΔM15* Tn*10* (tet^r)]

2.2.1 Maintainence and Storage of Bacterial Strains and Phage

All bacterial strains and phage stocks were maintained, cultured and stored using standard protocols (Miller, 1987).

2.2.2 Preparation of Plasmid DNA

Small amounts of plasmid DNA was prepared by extraction of DNA from a bacterial culture using the rapid-boil technique according to Sambrook *et al.*, (1989a) or with the Magic Miniprep DNA Purification System™(Promega). Large quantities of DNA were obtained by alkaline lysis of the bacteria followed by caesium chloride

centrifugation (Sambrook *et al.*, 1989a) or Magic/Wizard Maxiprep DNA Purification Systems™ (Promega).

2.2.3 Preparation of Phage DNA

Phage DNA was prepared using either plate lysate or liquid lysate methods (Sambrook *et al.*, 1989a; Ausubel *et al.*, 1989).

2.2.4 Digestion of DNA with Restriction Endonucleases

All restriction endonuclease digestion of DNA was performed using the conditions and buffers recommended by the supplier in sterile 1.5 ml microcentrifuge tubes. Digestions were carried out routinely for one hour at 37°C. Where double digestions containing incompatible buffer conditions occured, either ethanol precipitation of DNA between digestion steps or a 1x TA buffer (33 mM tris-acetate pH 7.8, 6.6 mM potassium acetate, 1 mM magnesium acetate, 0.05 mM β-mercaptoethanol, 5 µg/ml BSA) was used.

2.2.5 Agarose Gel Electrophoresis of DNA

Electrophoresis of DNA fragments was performed in low electroendosmosis grade agarose containing ethidium bromide (0.5 µg/ml) and either 1x TAE (0.04 M Tris, 0.02 M acetate, 0.001 M EDTA pH 8.0) or 1x TBE (0.09 M Tris, 0.09 M boric acid, 0.2 M EDTA pH 8.0) buffers (Sambrook *et al.*, 1989a). Molecular size markers were used to determine the approximate size of DNA fragments (Bethesda Research Laboratories or Promega Corporation). Unless otherwise stated, 1% agarose gels were used to separate DNA fragments. When DNA bands were purified after electrophoresis, 1% low melting point gels such as SeaPlaque™ and NuSieve™ (FMC) were used.

2.2.6 Purification of Fragments from Agarose Gels

DNA bands separated by gel electrophoresis were excised under illumination by long wavelength (366 nm) UV light. Several techniques were employed to purify DNA fragments embedded in agarose. These included freeze-squeeze (Thuring *et al.*, 1975), Geneclean™ (Bio 101) and Gelase™ (Epicentre). The protocol used depended upon the size of the fragment to be purified and was performed according to the suppliers instructions.

2.2.7 Transformation of Competent Cells

Competent XL-1 Blue cells suitable for transformation were prepared using calcium chloride and transformed by heat shock according to Sambrook *et al.* (1989a). While at Ohio State University, competent cells were obtained from Stratagene and used as recommended by the supplier.

2.2.8 Labelling DNA Probes with ^{32}P

DNA probes were labelled with [$\alpha^{32}P$]–dCTP using the Random Primers DNA labelling system (Bethesda Research Laboratories, MD, USA) or the Prime-A-Gene Labelling system (Promega Corporation, WI, USA) as directed by the manufacturers instructions. Radioactively labelled probes were purified using NucTrap™ push columns as described by the manufacturer (Stratagene, La Jolla, CA, USA). The specific activity and degree of incorporation of all radioactively labelled DNA probes were determined by scintillation counting in a Beckman LS8000 scintillation counter.

2.2.9 Hybridisation using DNA Probes

Prehybridisation

All colony lifts and Southern blots were treated in the same manner. Baked filters were prehybridised for 2-3 hours in a rotary oven or shaking incubator at an appropriate hybridisation temperature in 100-200 ml of prehybridisation solution [6X SSC containing 0.05% sodium pyrophosphate, 5X Denhardts [1% ficoll (Type 400), 1% polyvinylpyrrolidone, 1% bovine serum albumin (Fraction V)], 0.5% SDS].

Hybridisation.

Hybridisation was performed in either a rotary incubator or a shaking water bath at an appropriate temperature (usually 68°C) overnight. Probes were added at a concentration of $1x10^5$ cpm/μg DNA per ml of hybridisation solution. All DNA probes were boiled for 5 minutes and placed on ice before addition to the prehybridized lifts or blots.

Washing

Hybridisation solution was discarded and the filters washed twice [6x SSC, 1% SDS] for 1 hour at the hybridisation temperature. This was followed by two washes for 30 minutes in 6x SSC, 0.5% SDS at the same temperature. A final high stringency wash was performed for exactly 30 minutes in 1x SSC which had been pre-warmed to the temperature at which hybridisation had been performed.

2.2.10 Autoradiography

All filters and blots were wrapped in Gladwrap or Saranwrap™ to prevent drying out during exposure to X-ray film in autoradiography cassettes. Where necessary, autoradiography was carried out in the presence of intensifying screens at -70°C. Films were developed using an automated Kodak X-Omat processor or manually using Kodak developer and fixer.

2.2.11 Southern Transfer

DNA fragments separated by gel electrophoresis were transferred to nitrocellulose or nylon membrane by capillary transfer according to Southern (1975) or as described by the supplier of the transfer membrane.

2.2.12 Isolation of Genomic DNA

Preparation of total leucocytes

High molecular size DNA was prepared from white cells isolated from bovine blood samples which contained citrate as the anticoagulant. Red cells were lysed by a 10 minute incubation with 3 volumes of 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA at 0°C. The white cells were collected by centrifugation (160 x g for 10 minutes) and resuspended in 0.83% NH_4Cl in 10 mM HEPES, pH 7.0 for seven minutes at 37°C. The cells were again collected by centrifugation (650 x g for seven minutes) and washed with saline to remove residual red cells. The final pellet was collected by centrifugation at 650 x g for 10 minutes.

Isolation of total genomic DNA

The cell pellet was gently resuspended in TE buffer pH 8.0 and incubated at 50°C in 62.5 mM EDTA, 250 µg/ml Proteinase K and 0.625% Sarkosyl for 3-4 hours with occasional mixing. The lysed cells were extracted three times with tris-saturated phenol prior to overnight dialysis against 4 litres of dialysis buffer (50 mM Tris pH 8.0, 10 mM EDTA, 10 mM NaCl). The buffer was changed twice during this time. The dialysate was incubated for three hours at 37°C with heat-treated RNase (100 µg RNase/ml) and then extracted with phenol as described above. The DNA was precipitated by the addition of 0.3 M sodium acetate pH 6-7 and two volumes of 95% ethanol. High molecular size DNA was spooled from solution, washed in 95% ethanol (-20°C) and resuspended in TE buffer pH 8.0.

2.2.13 Digestion of Genomic DNA

Digestion of genomic DNA was carried out in sterile 1.5 ml microcentrifuge tubes according to the protocol outlined below.

Genomic DNA	~20 µg
React® buffer	20 µl
Sterile water	up to 200 µl
Restriction enzyme	4 U/µg DNA

Reactions were incubated at the appropriate temperature for approximately two hours. An aliquot (5 µl) was analysed by agarose gel electrophoresis to determine the

efficiency of the digestion. Reactions which required further digestion were incubated with additional restriction enzyme (2 U/μg DNA) for 2-4 hours. If further digestion was required, such as with Bam HI, additional restriction enzyme (2 U/μg DNA) was added and the reaction volume was doubled, to eliminate adverse glycerol concentrations. The correct proportion of React™ buffer was maintained within these reactions.

2.2.14 Electrophoresis of Genomic DNA

Prior to electrophoresis, genomic DNA was concentrated after digestion by ethanol precipitation according to Sambrook *et al.* (1989c). Samples were resuspended in a small volume of sterile TE buffer pH 8.0 and heated to 65°C for 10 minutes with loading dye before electrophoresis in 0.7% TBE agarose gel. Gels were electrophoresed for ~36 hours at ~35 V or until the dye front was ~4 cm from the end of the gel tray. The DNA fragments were stained with ethidium bromide (0.5 μg/ml), visualised and photographed under UV illumination.

2.2.15 Screening Bacteriophage Library

All bovine genomic libraries were screened as described by the supplier or according to Sambrook *et al.*, 1989a. The first round of screening was performed with a bacteriophage density of approximately 50,000 plaque forming units (pfu) per plate. This was decreased to approximately 200-300 pfu for second round screening and further decreased to approximately 80 pfu per plate for subsequent rounds of screening. Dilutions of phage stocks for plating were prepared in SM buffer (0.1 M NaCl, 8 mM $MgSO_4$, 20 mM Tris-HCl pH 7.5, 0.1% gelatin) immediately prior to use. Replicas of the λ plaques were transferred to nitrocellulose filters which were subsequently hybridised with ^{32}P-labelled DNA probes (Sambrook *et al.*, 1989a).

2.2.16 Size Determination of DNA Fragments

The electrophoretic mobility of linear double stranded DNA molecules passing through an agarose gel, is inversely proportional to the $\log_{10}$ of the number of base pairs contained within the fragment (Sambrook *et al.*, 1989a). Using this relationship, a graph of the distance migrated by DNA fragments of known molecular size through an agarose gel was plotted and used to estimate the size of other DNA fragments. A typical standard curve is shown in figure 4. In general, either the 1 kb BRL DNA ladder or a Hind III/Eco RI digest of λ DNA (Promega) were used as standards to construct the curves. This method was used routinely for estimating the size of digest fragments generated by restriction endonuclease analysis.

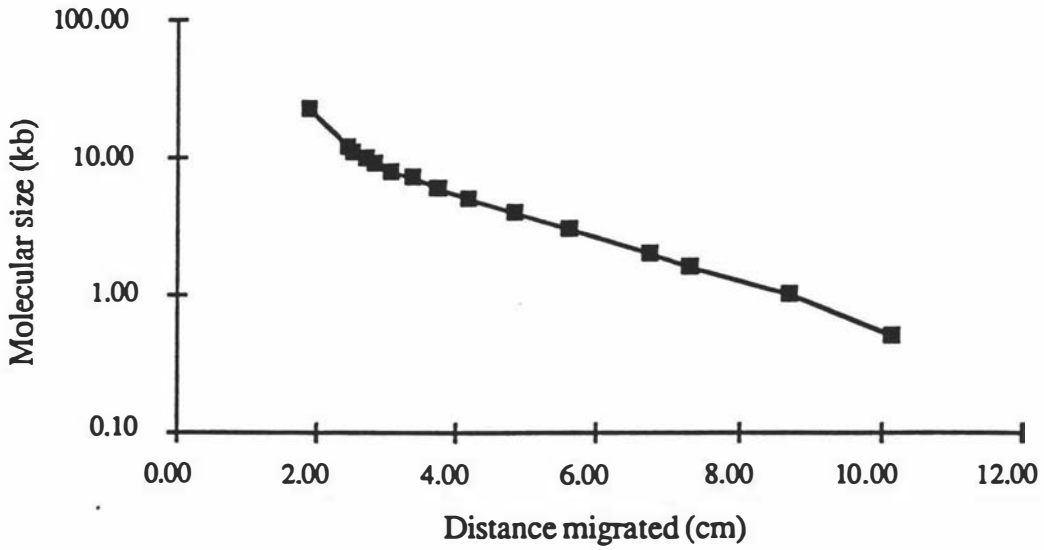


Figure 4: Standard molecular size curve

Relationship of DNA molecular size to electrophoretic mobility during agarose gel electrophoresis. Standard DNA fragments (Hind III/Eco RI digest λ DNA (Promega) or the BRL 1 kb ladder) were separated by electrophoresis in a 0.7% agarose gel as described in section 2.2.5.

2.2.17 DNA Amplification

Specific DNA sequences were amplified by PCR using the protocol recommended by Cetus corporation Ltd. Reactions were carried out in 0.5 ml sterile microcentrifuge tubes and overlayed with mineral oil to eliminate evaporation during the cycling of the reactions. All pipetting procedures were carried out using barrier tips to help avoid aerosol contamination of reactions. Typically, reactions consisted of 1x reaction buffer (supplied by manufacturer of polymerase), 2 mM $MgCl_2$, 3 mM deoxynucleotide triphosphates, 4-50 pmol of each oligonucleotide primer, 1-10 ng of template DNA and 2.5 U *Thermus aquaticus* (*Taq*) DNA polymerase. Plasmid DNA prepared by the rapid boil method (Sambrook *et al.*, 1989a) was diluted 100 fold to reduce the concentration of any potential inhibitors before the addition of ~2 µl to the PCR reaction. Reactions were performed in a DNA thermal cycler programmed for an initial 5 minute denaturation at 95°C followed by 30 cycles consisting of denaturation at 95°C for 1 minute; annealing for 1 minute at 65°C-55°C, depending upon the temperature at which the individual oligonucleotide primers gave a specific product; and extension at 72°C based upon 1 minute for each kilobase to be synthesised. Reactions were stored at 4°C prior to analysis. *Taq* DNA polymerase was used in all polymerase chain reactions used to amplify DNA sequences for DNA sequencing and the production of DNA probes. *Pfu* (*Pyrococcus furiosus*) DNA polymerase, a high-fidelity thermostable enzyme, was used to amplify DNA sequences for use in reporter gene studies. Routinely ~1.75 units were used in a single *Pfu* DNA polymerase chain reaction.

2.2.18 DNA Sequencing

Direct sequencing of both strands of DNA amplified by PCR was routinely used to determine the DNA sequence of clones and expression constructs. Sequences of interest were amplified by PCR using one biotinylated and one non-biotinylated primer in a volume of 50 µl. An aliquot (5 µl) of each reaction was analysed by agarose gel electrophoresis to determine the presence of the appropriate products. 40 µl of the PCR reaction was mixed with streptavidin paramagnetic M-280 dynabeads and the strands were separated by alkali treatment according to the manufacturers instructions. Single and double stranded templates were sequenced by the dideoxy chain termination method originally developed by Sanger *et al.* (1977) using Sequenase version 2.0 (9th Ed., USB, OH, USA). Sequencing gels were prepared and electrophoresed as described in Sambrook *et al.* (1989b). Dried gels were autoradiographed overnight at room temperature. Autoradiographs were developed using an automatic Kodak X-Omat developer. The DNA sequences were read manually and were analysed on a Vax 750 computer using the University of Wisconsin Genetics Computing Group (UWGCG) package (Deveraux *et al.*, 1984).

2.2.19 Expression of Reporter Gene Constructs (Ohio State University)

Expression experiments at Ohio State University were carried out by Dr C. Bennett and will be described briefly.

Culture of Mammary Cells

Primary bovine mammary epithelial cells and acini were prepared from lactating mammary tissue by enzymatic dissociation with collagenase, elastase, hyaluronidase, and chymotrypsin according to Talhouk *et al.* (1993). For culture, the cryopreserved mammary cells were thawed at 37°C and plated onto type I collagen gels prepared from rat tail tendons in the presence of M199 media containing prolactin (1 µg/ml) and in the absence of serum (Talhouk *et al.*, 1990). The co-culture of primary mammary acini and epithelial cells with collagen allows the cellular differentiation, synthesis and secretion of major milk proteins *in vitro* for 10-14 days (Talhouk *et al.*, 1993).

COMMA-1D cells, an epithelial cell line derived from mammary tissue of BALB/c mice in the middle of pregnancy, were grown on plastic as described by Danielson *et al.* (1984) and were transfected at day 4 post-plating.

Both the primary bovine mammary and the COMMA-1D cells were cultured at 37°C in a standard CO_2 tissue-culture incubator in a 5% CO_2 atmosphere.

Transfection of cultured mammary cells

Cultured mammary cells were transfected using procedures optimized for maximal subsequent expression of the control vectors containing the human growth hormone reporter gene controlled by the cytomegalovirus promoter, pCMV/hGH. Cells

were transfected in duplicate with ~1 μg of plasmid DNA complexed with DEAE-dextran, poly-ornithine and poly-glutamate-histamine in the presence of an osmotically balanced, buffer solution. Media was collected every two days.

Analysis of spent media

Media collected from microtitre plates was stored at -80°C prior to analysis. Lactoferrin and human growth hormone concentrations were measured by ELISA.

2.2.20 Expression of Reporter Gene Constructs (Massey University)

Culture of cells

COS cells, a generous gift from Dr K. Loomes, were maintained on Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum (FBS) containing penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were grown in 75 cm^2 flasks at 37°C in a 5% CO_2 atmosphere with the caps loosened to allow the passage of air. All manipulations of cells were performed in a sterile laminar flow hood. The media was changed every two to three days.

Preparation of media

DMEM media was prepared according to the manufacturers instructions and filter sterilised using a 0.2 μm Acrocap™ (Gelman Sciences, MI, USA) filter in a laminar flow hood. Antibiotics (100x stocks) and fetal bovine serum were purchased from Life Technologies Inc., MD, USA. Media was stored at 4°C and warmed to ~37°C before addition to cells.

Passage of cells

Cells were passed to new flasks or plates at approximately 80% confluency as judged by microscopic examination. Media was aspirated from the adherent cells and the cells were rinsed briefly with 2 ml of a 0.25% trypsin, 1 mM EDTA solution. A 4.5 ml aliquot of the trypsin/EDTA solution was pipetted onto the cells and incubated for 2-5 minutes within the laminar flow hood to release the cells from the surface of the flask. During this time, 5 ml of media was added to a 15 ml sterile conical tissue culture tube. The released cells were resuspended with a sterile pasteur pipette, transferred to the conical tube and collected by centrifugation at ~250 x g for five minutes. The cells were gently resuspended in ~2 ml of fresh sterile media and ~1 ml of the suspended cells were added to 75 cm^2 flasks containing 14 ml of media. The flasks were returned to the incubator and the cells allowed to adhere to the surface of the flasks. Cells which were being passed for transfections were added (~0.2-0.3 ml) to 4.5 ml of media in 60 mm sterile plates and incubated overnight prior to transfection.

Freezing of cells

Cells were frozen at regular intervals to maintain viable cell stocks. Passaged cells were resuspended in fetal bovine serum containing 10% DMSO. The resuspended cells were dispensed in 1 ml aliquots into cryotubes and chilled slowly to -70°C overnight. The following day, the tubes were transferred to liquid nitrogen were they were stored until required.

Transfection

Cells were transfected according to the CellPhect transfection kit method (Pharmacia, LKB Biotechnology, Uppsala, Sweden). Buffers were prepared and checked for the formation of a precipitate before sterilisation through a 0.2 μm filter. COS cells, ~80% confluency, were passed the day before the transfection from flasks into 60 mm plates. All transfection reactions were performed in duplicate. The β-galactosidase vector, pCH110, was included in all transfection reactions as an internal marker for monitoring and normalising expression results. The pGL2-Control (5 μg) vector (Promega) was used to evaluate the efficiency of the luciferase reporter assay. pGL2-Enhancer which lacks a functional promoter, was used to establish background expression levels. The transfection mixture was pipetted onto the surface of the media and the cells were returned to the tissue culture incubator and left overnight (~16-18 hours). The following morning the media was removed, the cells were washed twice with sterile PBS and fresh media was added. The cells were incubated for a further 36 hours before being harvested and assayed according to the luciferase assay system (Promega Corporation, WI, USA). β–Galactosidase was assayed according Herbomel *et al.* (1984). Luciferase activity was normalised against β-galactosidase activity for variable cell numbers and transfection efficiencies.

Chapter Three: Results and Discussion

3.1 Analysis of Clones Isolated from a Clonetech Genomic Library

A Clonetech bovine genomic library had been screened previously with a 394 bp probe derived from bovine lactoferrin cDNA sequences exon III-V (Figure 3). Three putative bovine lactoferrin clones (designated I, II and III) had been isolated. Different restriction digestion patterns indicated that these clones contained non-identical inserts (Figure 6A).

Following the isolation of the complete bovine lactoferrin cDNA, a 198 bp fragment corresponding to exons I and II (Section 2.1) was used as a probe to identify clones representing sequences 5' of the coding region. Hybridization of this probe to the digestion profiles of clones I-III (Figure 6A) showed that clone I contained sequences which were complementary to the probe (Figure 6B). Specific hybridization signals were not detected within the clone II or clone III digestion profiles, indicating that neither of these clones contained bovine lactoferrin sequences corresponding to exon I or exon II. The ~1.6 kb fragment of the 1 kb DNA ladder also hybridised to the radiolabelled probe. This was observed as a faint band on the autoradiograph in figure 6B, lane 1.

Cleavage of clone I with Eco RI resulted in a ~2 kb fragment which hybridized to the 198 bp cDNA probe. This fragment was subcloned and a partial restriction map was produced (Figure 5). Sequence analysis indicated that this fragment was likely to contain 1511 bp of intron I, all of exon II and 350 bp of intron II (Appendix 1). Exon II was located within a 531 bp Bam HI fragment which was used as a hybridisation probe in later investigations (Figure 5). A single C to T base substitution was identified by comparison of this sequence with the bovine lactoferrin cDNA sequence (Appendix 2).

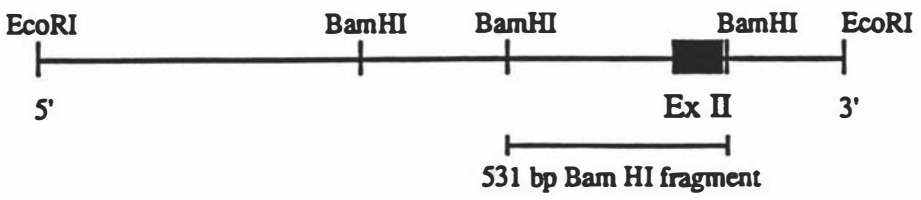


Figure 5: Partial restriction map of a ~2 kb Eco RI fragment isolated from clone I

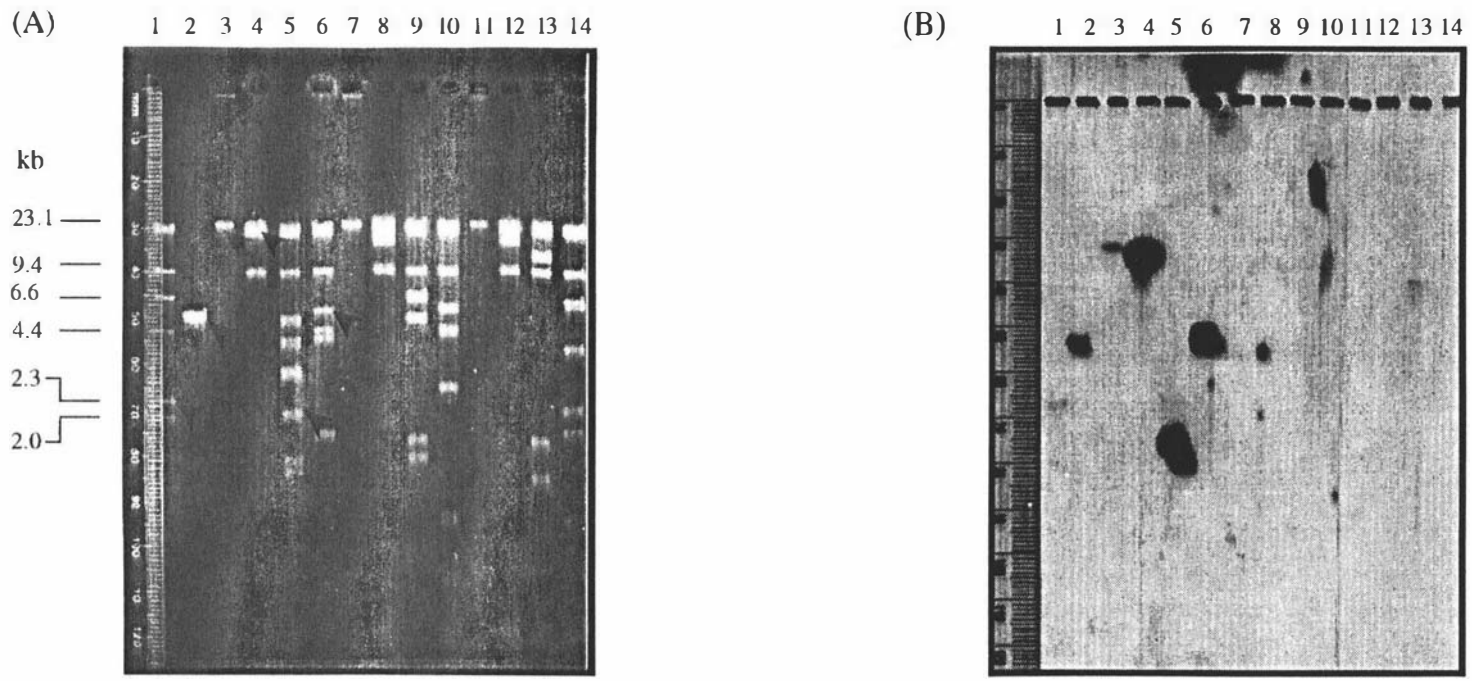


Figure 6: Restriction analysis and Southern hybridisation of clones isolated from a Clonetech genomic library

(A). Gel photograph of isolates I, II and III after cleavage with restriction endonucleases. Arrowheads indicate fragments that hybridised to the probe.

(B). Autoradiograph of Gel (A) hybridised to a radiolabelled probe comprising of exon I and exon II sequences. The ~2.3 kb fragment of the λ Hind III ladder gave a non-specific hybridsation signal.

(1). λ Hind III markers; (2). Linearised BLf cDNA; (3). Uncut Clone I DNA; (4). Clone I cleaved with Sal I ; (5). Clone I cleaved with Sal I/Eco RI; (6). Clone I cleaved with Sal I/Sst I; (7). Uncut Clone II; (8). Clone II cleaved with Sal I; (9). Clone II cleaved with Sal I/Eco RI; (10). Clone II cleaved with Sal I/Sst I; (11). Uncut Clone III; (12). Clone III cleaved with Sal I; (13). Clone III cleaved with Sal I/Eco RI; (14). Clone III cleaved with Sal I/Sst I.

An oligonucleotide (Te1) designed from the 5' most end of the bovine lactoferrin cDNA sequence (Section 2.1) was used as a probe to investigate the presence of bovine lactoferrin sequences corresponding to exon I within these clones. No specific hybridization signals were detected (data not shown), indicating that these clones did not contain sequences representing exon I of the bovine lactoferrin gene. Because the DNA sequences 5' to exon I were the primary focus of this study, these clones were not investigated further.

The library was rescreened with the genomic BamHI fragment of 531 bp (Figure 5) and resulted in the re-isolation of clone I. The Clonetech library was a commerical library which had been amplified prior to sale. Because recombinant clones frequently give rise to plaques of unequal sizes, amplification of libraries can result in a distortion in the relative frequency of clones within the library. This can lead to the preferential amplification of some recombinants and the loss of others from the library. Consequently, unamplified libraries are considered to be a more accurate representation of the total genomic sequences.

An unamplified bovine genomic library was subsequently provided by Professor Floyd Schanbacher of Ohio State University. Genomic sequences corresponding to the 5' region of bovine lactoferrin were more likely to be isolated from this library than the amplified library from Clonetech. Consequently, screening of the Clonetech library was discontinued.

3.2 Preparation of probes for screening

Part of the cDNA sequence (2165 nt) of bovine lactoferrin had been determined previously (Mead & Tweedie, 1990). Alignment of the bovine lactoferrin and serum transferrin amino acid sequences indicated that these related proteins were likely to have a similar gene arrangement. This was the basis for the assignment of the bovine lactoferrin exon regions within the cDNA sequence and therefore the determination of which exons were likely to be located within the sequences used to screen the phage libraries.

An exon I probe was isolated from cDNA sequence contained in the plasmid p5'-350 bLf (Figure 7). Double digestion of this plasmid with the restriction endonucleases Asp I and Eco RV, produced a 103 bp exon I fragment and a 437 bp fragment representing exon II-IV. An exon II probe of 531 bp, was prepared by digesting the 2.025 kb fragment, isolated from the Clonetech genomic library (Figure 5), with the restriction endonuclease Bam HI.

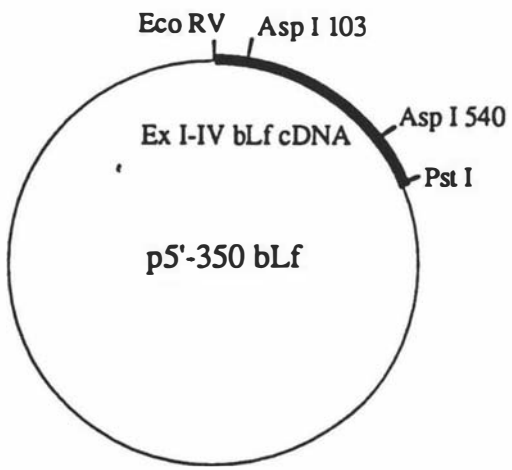


Figure 7: Diagram of p5'-350 bLf plasmid

p5'-350 bLf consisted of the first 697 bp of the bovine lactoferrin cDNA sequence corresponding to exons I-IV cloned into the Eco RV and Pst I restriction sites of pGEM-5Zf+ (Promega). This clone was a gift from S.Pattanajitvillai.

3.3 Screening the Stratagene bovine genomic library

A λFIX II unamplified genomic library was screened for 5' bovine lactoferrin sequences using a probe comprising of exon I sequences and also a probe derived from exon II. The λ FIX® II library was plated on 150 mm NZY agar petri dishes using the recommended MRA(P2) host strain (Stratagene). A total of approximately 1.1 x 10^6 plaques were screened. Duplicate lifts were taken (Sambrook *et al.*, 1989a) and hybridised separately to a cDNA probe specific for exon I or a genomic probe comprising exon II sequences.

The first round of screening with an exon I probe produced a high background combined with weak hybridisation signals (Figure 8). The background signals suggested that not all of the hybridisation signals resulted from specific interactions. Twenty agar plugs corresponding to the putative positive signals from this screening were picked for a second round of screening with the exon I probe (~300 pfu/plate). Three of the twenty plugs selected in the first round gave clear, superimposable, signals on duplicate lifts (Figure 8). The three clones were rescreened using the exon II probe and all three gave positive signals. Therefore, these clones contained sequences corresponding to both exon I and exon II.

The filters which had been hybridised with the exon II probe in the initial round of screening gave distinct signals with a low background, allowing 24 plugs to be selected for subsequent screening analysis. It was not possible to compare putative exon I and exon II positive signals obtained from the first round of screening due to varying background signals. Further screening of the exon II positive plugs with an exon I probe produced no detectable hybridisation signals, suggesting that these clones did not contain exon I regions. Consequently, these clones were stored at 4°C.

The clones which were selected with the exon I probe were derived from a single plug picked from the first exon I screening. For this reason, it was likely that the three positive clones were identical. Consequently, only one clone was selected for subsequent investigation. Large quantities of phage DNA was prepared from this clone (called λOHNZ1) by either the plate lysis or liquid lysate method (Sambrook *et al.*, 1989a; Ausubel *et al.*, 1989).

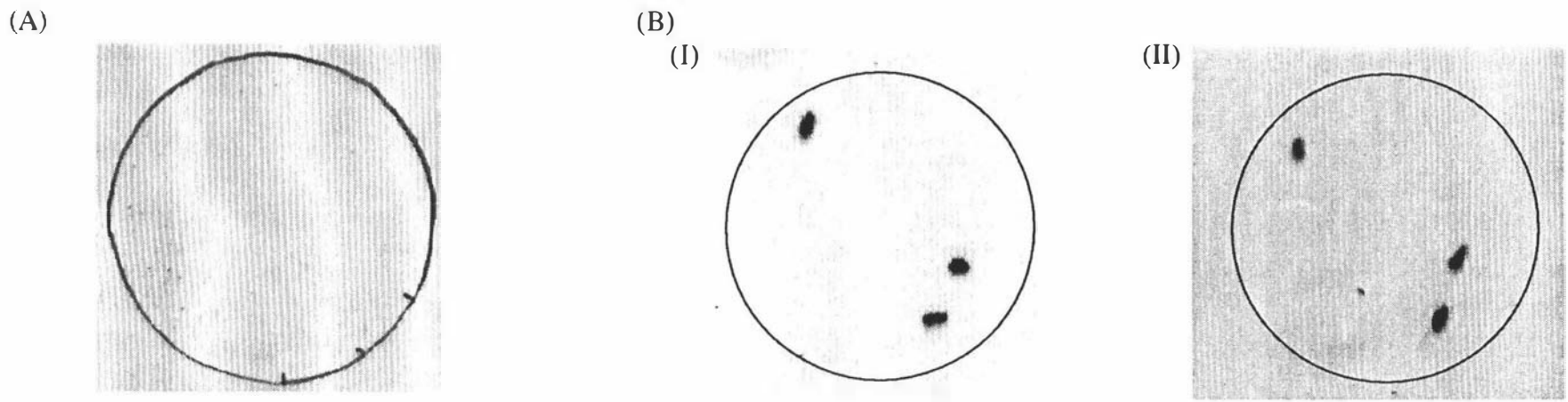


Figure 8: Autoradiographs of hybridisation filters used in the screening of the λFIX II library.

The library was plated and lifts prepared as described in section 3.3. The filters were hybridised to a radiolabelled probe derived from bovine lactoferrin exon I sequences, washed under conditions of high stringency (1X SSC, 68°C), and autoradiographed.

(A). First round filter screened with an exon I probe showing weak hybridisation signals and high background signals.

(B). Second round duplicate lift filters (I and II) hybridised with an exon I probe showing clear, superimposable signals.

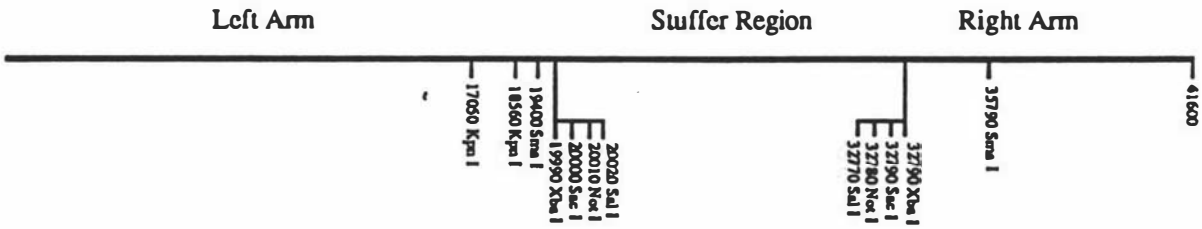
3.4 Characterisation of λOHNZ1 by restriction endonuclease mapping

λOHNZ1 was characterised by restriction endonuclease mapping. Restriction enzymes, either alone or in various combinations, were used to digest λOHNZ1 DNA. Following separation by gel electrophoresis and Southern transfer, the fragments were hybridised with either an exon I or an exon II ^{32}P-labelled probe in an attempt to interpret the complex fragmentation patterns produced by each digestion. The strategy used for the construction of the restriction map is outlined below. A detailed analysis and discussion of the data obtained from this investigation is provided in Appendix 3.

A map of the λFIX II cloning vector is shown in figure 9. This shows that Not I, Sst I and Xba I restriction sites flank the cloning junctions of the insert. Not I, a rare cutting restriction enzyme, was used to cleave the entire insert from the vector.

Figure 9: Map of λFIX II vector

Modified map of lambda FIX II cloning vector showing the restriction enzyme cleavage sites relevant to this investigation (Sambrook *et al.*, 1989a).



Digestion products were orientated by comparison of single digests to double digestions with Not I and the enzyme in question (Figures 10A and 13A). The sizes of all digestion fragments were deduced from a standard molecular size curve constructed from the molecular size markers included on each gel (Section 2.2.16). This information is summarised in tables 4 and 6. Fragments cleaved by Not I were identified and mapped to the appropriate regions of the clone. Southern hybridisation analysis was used to identify specific fragments which contained complementary sequences to either an exon I or an exon II probe (Figures 10B and 13B, Tables 5 and 7). This information is illustrated in figures 12 and 15. This process allowed the position of the Eco RI and Sma I restriction sites within λOHNZ1 to be deduced (Figures 11 and 14).

This approach was inappropriate for the Sst I and Xba I as the restriction sites for these enzymes are located adjacent to the Not I site within the vector (Figure 9) and

therefore produced identical digestion profiles for both single digests and double digests with Not I. Consequently, the Sst I and Xba I restriction sites within λOHNZ1 were orientated relative to other defined restriction sites, such as Eco RI and Sma I. Figure 16A shows the cleavage patterns produced by some of these restriction digestions. A summary of this information is shown in table 8, figure 17. The corresponding Southern blot identified fragments which hybridised to the probe derived from bovine lactoferrin exon I sequences (Table 9, Figure 16B). Figure 18 illustrates the fragments which hybridised to the probe.

The map of λOHNZ1 was further characterised by digesting λOHNZ1 DNA with the restriction enzymes Kpn I, Xho I and Hind III in conjunction with other mapped restriction enzyme sites (Figure 19, Table 10). These results are summarised in figure 20. Fragments which contained bovine lactoferrin exon I and exon II sequences were identified by Southern hybridisation (Table 11 and Figure 21). A summary of this data is shown in figures 22 and 23. Digestions of λOHNZ1 DNA with other combinations of restriction endonucleases produced digestion fragment sizes which supported the position of the mapped restriction sites within λOHNZ1 for several restriction endonucleases (Figures 24 and 25).

λOHNZ1 has a total size of ~46.5 kb. This suggested that the insert was ~17.3 kb in length. Southern hybridisation analysis indicated that the insert had been cloned in a 3' to 5' orientation relative to the left and right arms of the λFIX II vector. Exon I was located in a ~0.87 kb Sst I/Sma I fragment which was approximately 4.9 kb from the left arm cloning site. A ~0.81 kb Eco RI/Xho I fragment positioned ~0.9 kb from the left arm of the vector hybridised to the exon II probe. The restriction sites of Not I, Eco RI, Xho I, Kpn I, Hind III and Sma I were defined (Figure 26). The Xba I and Sst I restriction sites within λOHNZ1 require further characterisation, particularly within the 5' most region of the clone. This would involve the isolation of a 5' fragment such as the ~8.0 Kpn I/Not I fragment, and subjecting this fragment to additional restriction analysis. This would be necessary to reduce the complexity of the digestion patterns and to allow the remaining cleavage sites of both the Sst I and Xba I restriction endonucleases to be defined. Bam HI cleaved λOHNZ1 several times, producing a complex digestion profile. The ~1.58 kb Bam HI fragment which hybridised to exon II has been positioned within the clone (Figure 27). However, the position of the ~0.62 kb Bam HI fragment within λOHNZ1 which hybridised to exon I, could not be located using the data obtained thus far. The precise location of the ~0.62 kb Bam HI fragment in the λOHNZ1 clone was established subsequently as described in section 3.5.1. Restriction endonuclease fragments which were subjected to additional analyses are shown in figure 27.

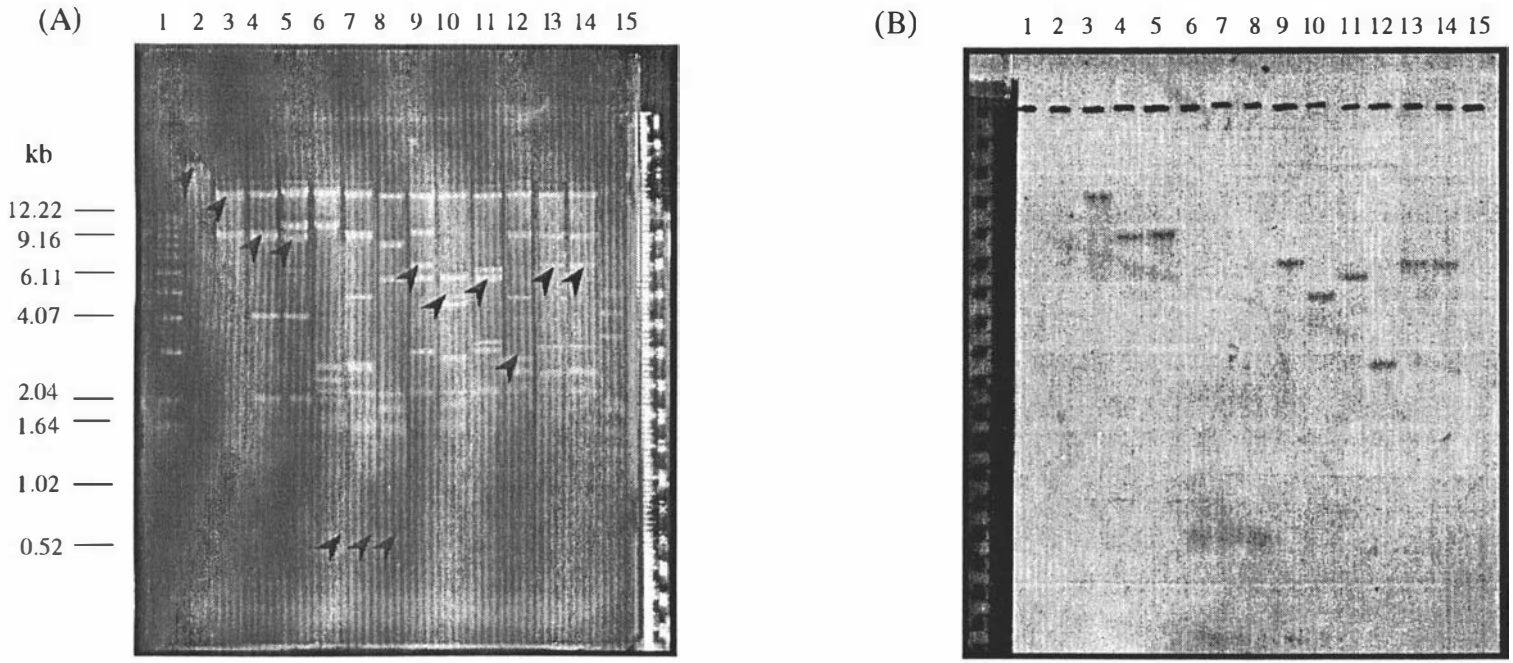


Figure 10: Restriction digestions of λOHNZ1 and autoradiograph

(A). λOHNZ1 DNA (~2 μg) was digested as described in section 2.2.4 with the restriction endonucleases listed below. The fragments were separated by electrophoesis through a 0.7% agarose gel in 1x TBE for 3-4 hours at ~70 V. After electrophoresis the fragments were stained with ethidium bromide and photographed prior to Southern hybridisation. Arrowheads indicate the fragments which hybridised to the probe. Weak hybridisation (arrow *) was observed with the uncut λOHNZ1 DNA.
(B). Digest fragments (A) were transferred to a nylon membrane and hybridised to a ^{32}P-labelled probe derived from exon I sequences. After the final wash (68°C, 1x SSC) the membrane was exposed to X-ray film and developed.

1. BRL 1 kb DNA ladder. 2. λOHNZ1 DNA ~500 ng (Uncut). λOHNZ1 DNA (~2 μg) digested with: 3. Not I; 4. Not I/Eco RI; 5. Eco RI; 6. Eco RI/Bam HI; 7. Not I/Bam HI; 8. Sma I/Bam HI; 9. Sma I; 10. Sma I/Eco RI; 11. Sma I/Not I; 12. Sst I/Not I; 13. Xba I; 14. Xba I/Not I. 15. Promega λ Eco RI/Hind III markers

Table 4: Deduced molecular sizes of digest fragments shown in figure 10A

The restriction endonuclease used in each digestion is shown at the top of each column. Fragments produced by partial digestion (*) are excluded from the total molecular size of each digestion profile shown at the bottom of the columns. All values are approximate and were deduced from a DNA standard curve constructed from the Promega λ Eco RI/Hind III and 1 kb BRL DNA ladder.

Not I (kb)	Not I/Eco RI (kb)	Eco RI (kb)	Eco RI/Bam HI (kb)	Not I/Bam HI (kb)	Sma I/Bam HI (kb)	Sma I (kb)	Sma I/Eco RI (kb)	Sma I/Not I (kb)	Sst I/Not I (kb)	Xba I (kb)	Xba I/Not I (kb)
20.00 17.30 9.10	20.00 9.10 8.80 4.17 2.00 1.20 0.98	21.00 10.30 8.80 6.20* 4.17 2.00	21.00 10.30 2.70 2.45 2.25 2.00 1.73 1.60 1.10 0.62	20.00 9.10 5.00 2.80 2.65 2.25 1.60 1.25 1.10 0.62	19.30 8.50 5.70 2.20 1.85 1.65 1.55 1.25 1.00 0.62	19.30 9.80 6.50 5.70 3.00 2.15	19.30 5.70 4.80 4.60 2.95 2.15 1.95 1.70 1.55 1.18	19.30 6.40 5.80 5.70 3.40 3.00 2.15 0.68	20.00 9.10 4.80 4.05* 2.80 2.60 2.25	20.00 9.10 6.60 3.20 2.60 2.20	20.00 9.10 6.60 3.20 2.60 2.20
46.40	46.25	46.27	45.75	46.37	43.62	46.45	45.88	46.43	41.55	43.70	43.70

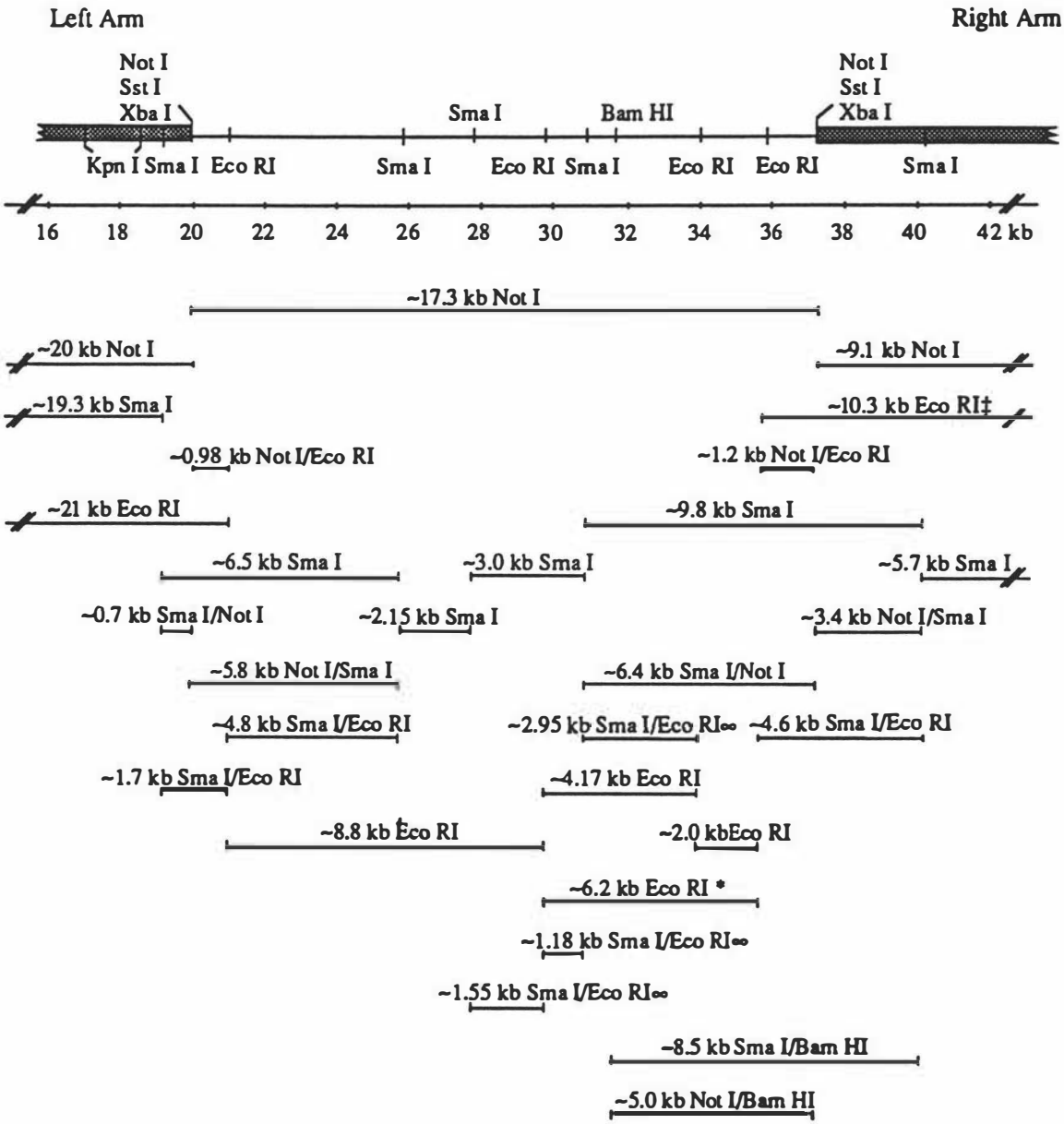


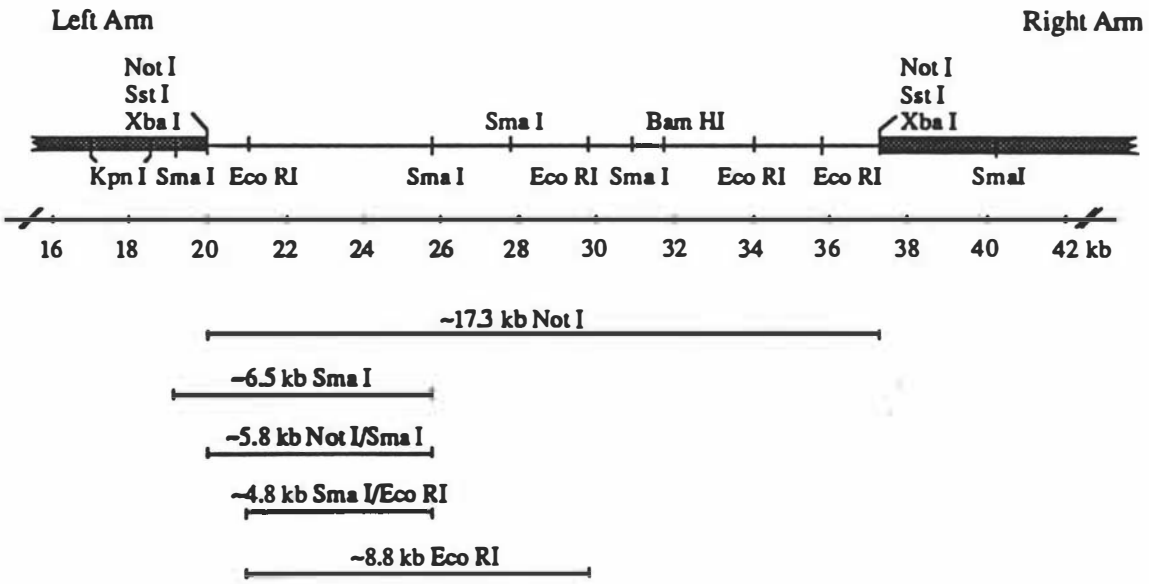
Figure 11: Partial restriction map of λOHNZ1

The ~10.3 kb Eco RI fragment is marked with a (‡) to indicate that this fragment was thought to be migrating anomalously. A ~6.2 kb partial digestion product (*) is also displayed. (∞) fragments which vary from the expected value deduced from the restriction map and the fragment size observed on the agarose gel (Figure 10A). These fragments are discussed in Appendix 3.

Table 5: Digestion fragments from figure 10 which hybridised to an exon I probe

Restriction endonucleasc	Molecular size of hybridisation fragment (kb)
Not I	~17.30
Not I/Eco RI	~8.80
Eco RI	~8.80
Eco RI/Bam HI	~0.62
Not I/Bam HI	~0.62
Sma I/Bam HI	~0.62
Sma I	~6.50
Sma I/Eco RI	~4.80
Not I/Sma I	~5.80
Not I/Sst I	~4.80
Xba I	~6.60
Not I/Xba I	~6.60

Figure 12. Partial restriction map of λOHNZ1 showing the location of fragments that hybridised to exon I



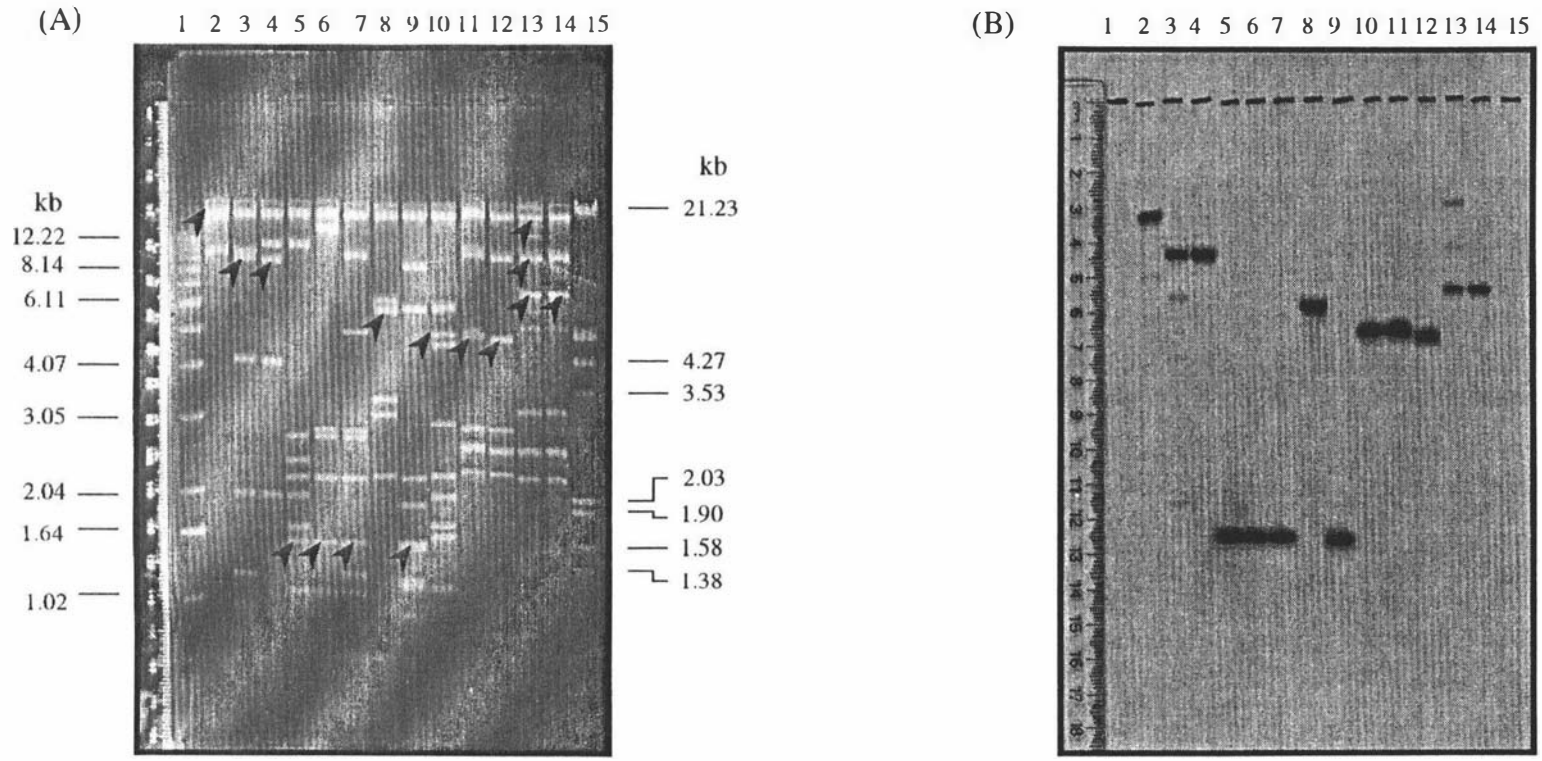


Figure 13: Agarose gel electrophoresis of digested λOHNZ1 DNA and autoradiograph of λOHNZ1 fragments hybridised to an exon II probe

(A). λOHNZ1 DNA was digested with the restriction endonucleases listed below for one hour at 37°C. The samples were separated by gel electrophoresis through a 0.7% agarose gel in 1x TBE and photographed under UV illumination. Arrowheads indicate the fragments which hybridised to the probe.
(B). Digested samples were subjected to electrophoresis (A) and immobilised on nylon membrane by Southern transfer. The membrane was hybridised overnight at 68°C and washed under conditions of high stringency (1x SSC, 68°C). The resultant blot was wrapped in Saranwrap™ and exposed to film at -70°C overnight with intensifying screens.

1. BRL 1 kb DNA Ladder. λOHNZ1 DNA (2 µg) digested with: 2. Not I; 3. Not I/Eco RI; 4. Eco RI; 5. Eco RI/Bam HI; 6. Bam HI; 7. Not I/Bam HI; 8. Sma I/Not I; 9. Sma I/Bam HI; 10. Sma I/Eco RI; 11. Sst I; 12. Sst I/Not I; 13. Xba I; 14. Xba I/Not I. 15. Promega λ Eco RI/Hind III markers

Table 6: Fragment sizes of digestion products shown in figure 13A

Fragments denoted with a (‡) were presumed to be ~10.3 kb as described Appendix 3. Partial fragments (*) were not included in the total size values. All values were approximate and deduced from a standard DNA molecular size curve.

Not I (kb)	NotI/Eco RI (kb)	Eco RI (kb)	Eco RI/Bam HI (kb)	Bam HI (kb)	Not I/Bam HI (kb)	Sma I/Not I (kb)	Sma I/Bam HI (kb)	Sma I/Eco RI (kb)	Sst I (kb)	Sst I/Not I (kb)	Xba I (kb)	Xba I/Not I (kb)
19.60 17.00 10.20	20.00 9.10 8.80 4.15 2.00 1.20 1.00	21.00 10.80‡ 8.80 4.15 2.00	21.00 10.80‡ 2.75 2.45 2.20 2.00 1.73 1.58 1.15 0.78 0.28	21.28 14.50 2.85 2.75 2.20 1.58 1.15 0.74	20.00 9.10 5.00 2.85 2.75 2.20 1.58 1.28 1.15 0.74	19.30 6.40 5.80 5.70 3.40 3.00 2.20 0.70	19.30 8.60 5.70 2.20 1.95 1.65 1.58 1.52 1.25 1.20 1.00 0.68	19.30 5.70 4.80 4.60 2.95 2.20 1.95 1.70 1.55 1.08	20.00 9.10 4.90 2.90 2.65 2.65 2.20 0.86 0.74	20.00 9.10 4.90 2.90 2.65 2.65 2.20 0.86 0.74	20.00 17.00* 14.00* 9.80* 9.10 6.60 5.40* 5.00* 3.60* 3.40* 3.20 2.65 2.65 2.25	20.00 9.10 6.60 5.40* 5.00* 3.60* 3.40* 3.20 2.85* 2.65 2.65 2.25
46.80	46.25	45.25	46.22	47.05	46.65	46.50	46.63	45.83	46.00	46.00	46.45	46.45

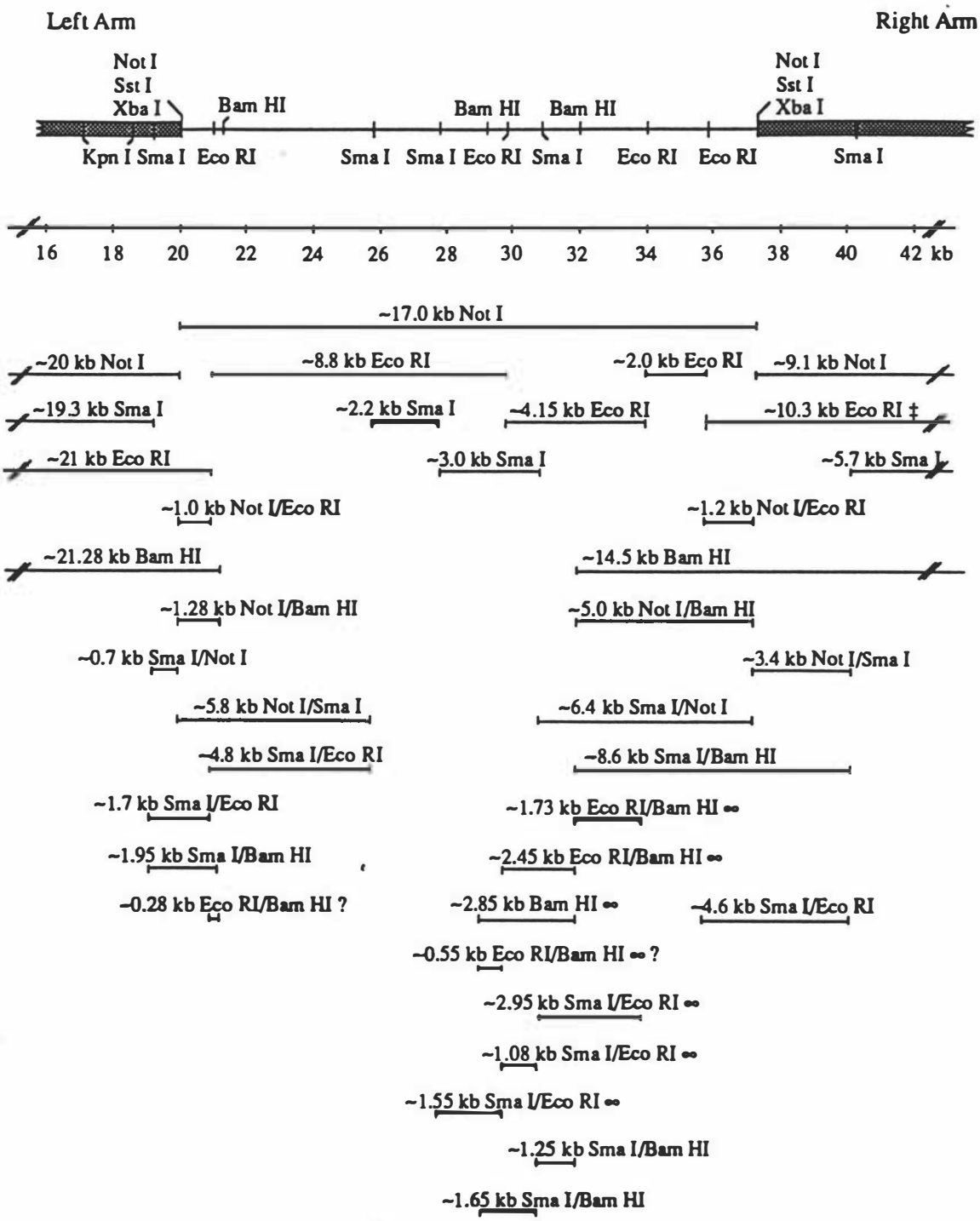


Figure 14: Relative position of restriction endonuclease fragments produced by digestion of λOHNZ1 as shown in figure 13A

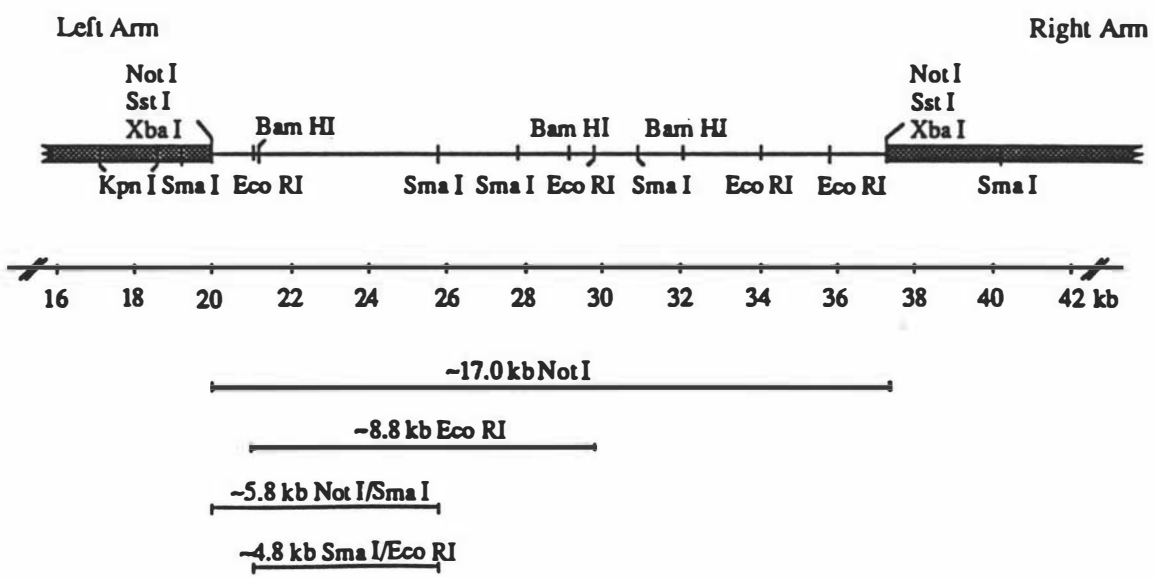
∞ Denotes fragment which have molecular sizes which vary from those expected or predicted from the restriction mapped sites. A ~10.3 kb Eco RI fragment (‡) represented a ~10.8 kb Eco RI fragment thought to be migrating anomalously. A ~0.28 kb Eco RI/Bam HI fragment (?) predicted from the mapped restriction sites, has been included for completeness. The position of an ambiguous ~0.55 kb Eco RI/Bam HI fragment (?) has been shown however this fragment has not been observed within the digestion profile.

Table 7: Fragment sizes of digestion products shown in figure 13 which hybridised to a radiolabelled probe derived from exon II sequences of bovine lactoferrin

Partial digest products (*) which also hybridised to the probe have been included.

Restriction endonuclease	Molecular size of hybridisation fragments (kb)
Not I	~17.00
Not I/Eco RI	~8.80
Eco RI	~8.80
Eco RI/Bam HI	~1.58
Bam HI	~1.58
Not I/Bam HI	~1.58
Sma I/Not I	~5.80
Sma I/Bam HI	~1.58
Sma I/Eco RI	~4.80
Sst I	~4.90
Sst I/Not I	~4.90
Xba I	~17.00*, ~9.80*, ~6.60
Xba I/Not I	~6.60

Figure 15: A diagrammatic summary of λOHNZ1 fragments which hybridise to exon II



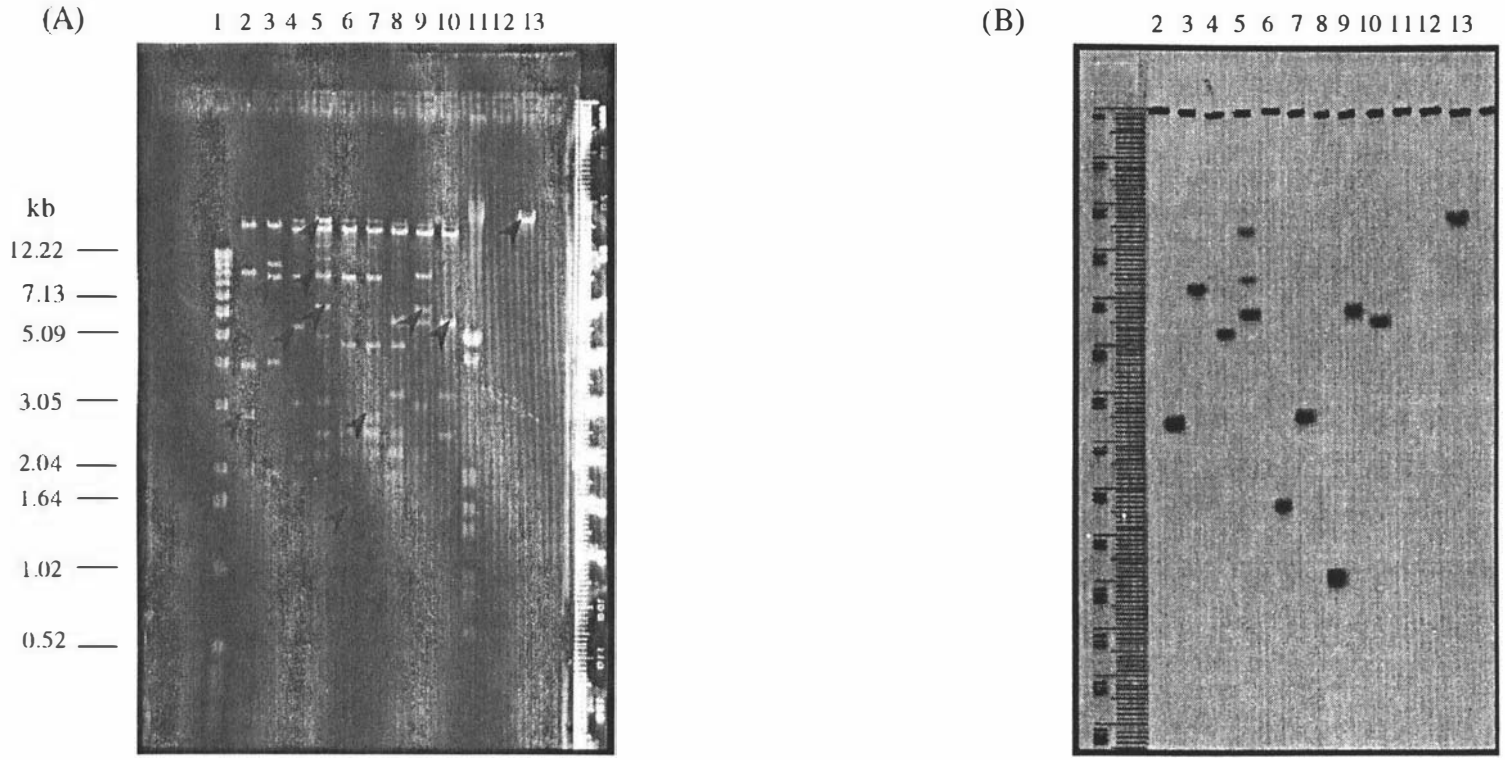


Figure 16: Gel photograph and Southern blot of λOHNZ1 digests

(A). ~2 µg of λOHNZ1 DNA was digested for 1 hour at 37°C with the restriction endonucleases listed below. Samples were separated by electrophoresis at ~70V for 3-4 hours in a 0.7% agarose gel in 1x TBE. The gel was stained in ethidium bromide and photographed under UV illumination. Arrowheads indicate fragments which hybridised.

(B). The separated digestion fragments (Figure 16A) were transferred to GeneScreen Plus nylon membrane, hybridized to a radiolabelled exon II genomic probe, washed (1x SSC, 68°C) and exposed to X-ray film at -70°C with intensifying screens.

1. BRL 1 kb DNA ladder. λOHNZ1 DNA (~2 µg) digested with: 2. Sst I/Eco RI; 3. Eco RI; 4. Xba I/Eco RI; 5. Xba I; 6. Sst I/Xba I; 7. Sst I; 8. Sma I/Sst I; 9. Sma I; 10. Sma I/Xba I; 11. Promega λ Eco RI/Hind III markers. 12. Blank. 13. Uncut λOHNZ1 DNA (~1 µg)

Table 8 : Total molecular size values of restriction endonuclease fragments of λOHNZ1 as shown in figure 16

Partial digestion products (*) have been excluded from the total molecular size values. All molecular size values are approximate and deduced from a standard DNA size curve.

Sst I/Eco RI (kb)	Eco RI (kb)	Xba I/Eco RI (kb)	Xba I (kb)	Sst I/Xba I (kb)	Sst I (kb)	Sma I/Sst I (kb)	Sma I (kb)	Sma I/Xba I (kb)
20.00 11.50* 9.10 3.85 2.85 2.05 1.55 1.30 1.10 0.76 0.59	21.00 10.30 8.80 4.20 2.20	20.00 15.30* 11.70* 10.50* 9.10 8.60* 5.40 3.05 2.15 1.85 1.28 1.18 0.96 0.35	20.00 18.00* 16.30* 12.20* 9.80* 9.10 7.30* 6.40 5.20* 4.90* 3.65* 3.20 2.65 2.65 2.20 0.20	20.00 14.80* 12.20* 9.10 4.90 2.65 2.20 1.90 1.50 1.25 0.75	20.00 9.10 4.90 2.85 2.65 2.65 2.20 0.76 0.59	19.30 12.80* 10.80* 5.70 4.90 3.40 2.65 2.25 2.25 2.00 0.87 0.76 0.70 0.59	19.30 12.80* 10.80* 9.80 6.50 5.70 3.00 2.10	19.30 12.80* 10.80* 5.80 5.70 3.40 2.65 2.65 1.70 1.50 1.20 1.00 0.70 0.60 0.20
43.15	46.50	45.32	46.40	44.25	45.70	45.37	46.40	46.40

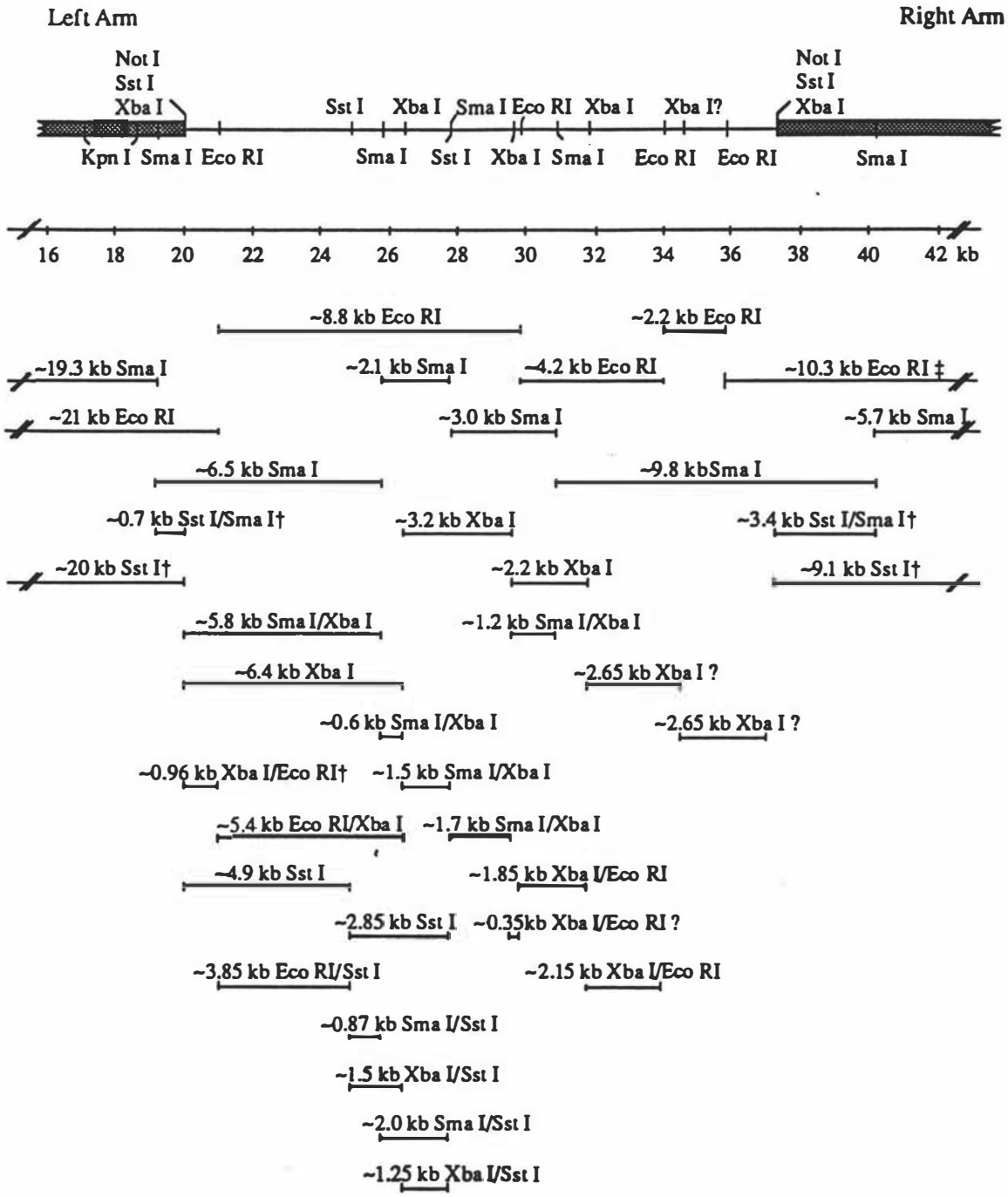


Figure 17: Schematic representation of restriction fragments showing the putative Sst I and Xba I cleavage sites within λOHNZ1

The ~10.3 kb Eco RI fragment (‡) represented a ~10.8 kb fragment which was thought to be migrating anomalously. (†) denotes fragments which were produced by digestions including Xba I or Sst I because these restriction sites are adjacent at the vector arms. A (?) indicates fragments which were observed within a digestion profile but the precise location of these fragments within the clone could not be determined.

Table 9: Digestion fragments from figure 16 which hybridised to a radiolabelled probe representing exon I sequences of bovine lactoferrin

Partial digestion products (*) which also hybridised have been indicated.

Restriction endonuclease	Molecular size of hybridisation fragment (kb)
Sst I/Eco RI	~2.85
Eco RI	~8.80
Xba I/Eco RI	~5.40
Xba I	~46*, ~9.80*, ~6.40
Sst I/Xba I	~1.56
Sst I	~2.85
Sma I/Sst I	~0.87
Sma I	~6.50
Sma I/Xba I	~5.80

Figure 18: Schematic restriction endonuclease map showing the digestion fragments which hybridised to an exon I radiolabelled probe

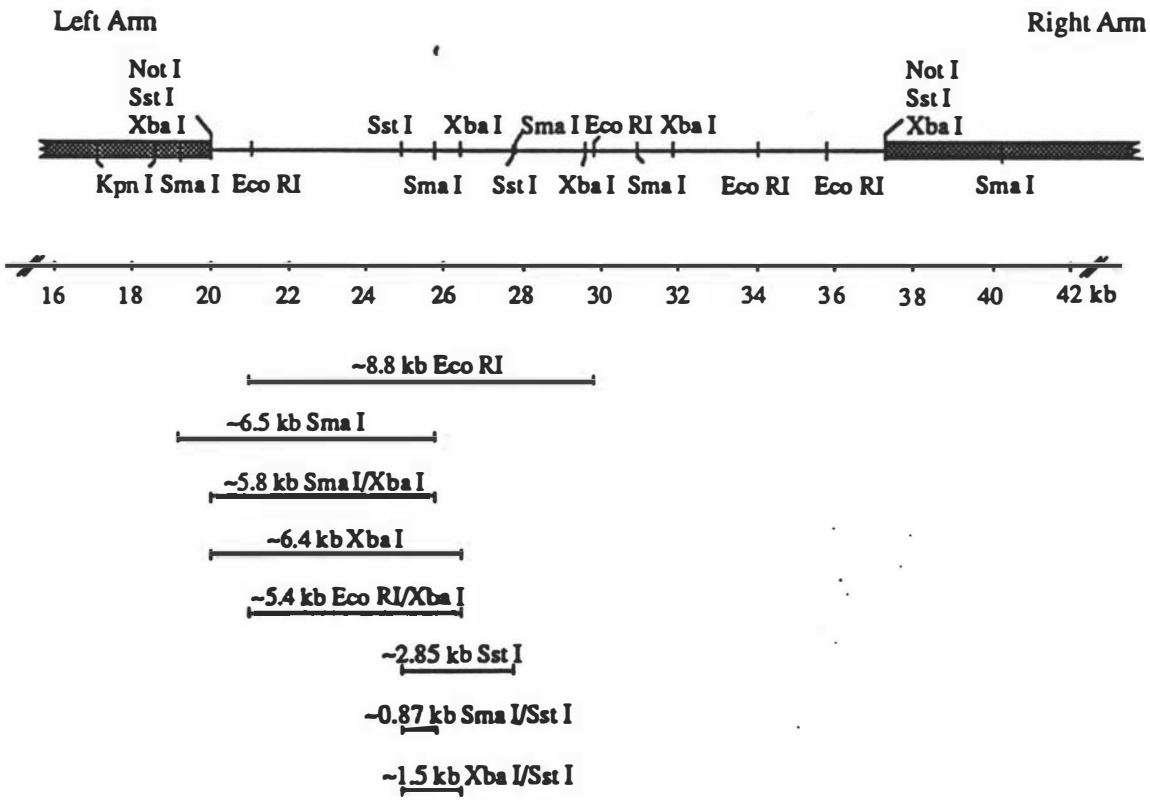


Figure 19: Restriction endonuclease analysis of λOHNZ1.

Samples of λOHNZ1 (~2 μg) were digested for one hour at 37°C with the restriction endonucleases listed below. Fragments were separated by electrophoresis through a 0.7% agarose gel in 1x TBE at ~70 V for 3-4 hours. The fragments were stained with ethidium bromide and photographed under UV illumination.

Lane 1.	BRL 1 kb DNA ladder
Lane 2.	λOHNZ1 DNA (~2 μg) digested with (Kpn I/Not I)
Lane 3.	" " " " " (Kpn I)
Lane 4.	" " " " " (Kpn I/Hind III)
Lane 5.	" " " " " (Hind III)
Lane 6.	" " " " " (Hind III/Eco RI)
Lane 7.	" " " " " (Eco RI)
Lane 8.	" " " " " (Xho I/Eco RI)
Lane 9.	" " " " " (Kpn I/Eco RI)
Lane 10.	" " " " " (Xba I/Kpn I)
Lane 11.	" " " " " (Xba I)
Lane 12.	" " " " " (Xba I/Eco RI)
Lane 13.	" " " " " (Xba I/Xho I)
Lane 14.	" " " " " (Xho I)
Lane 15.	BRL 1 kb DNA ladder

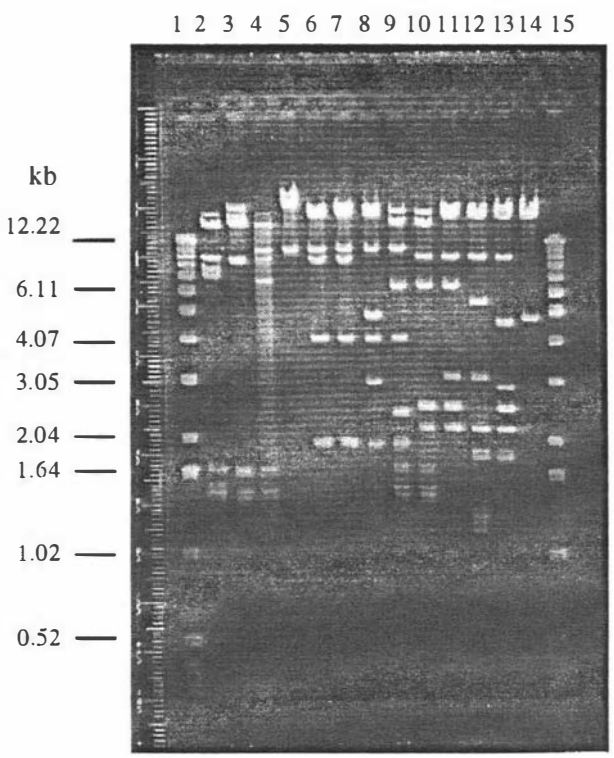


Table 10: Molecular sizes of digestion fragments deduced from figure 19

Each column contains the fragments produced by cleavage with the restriction endonuclease(s). The total molecular size is shown at the bottom of each column. All values are approximate and deduced from a standard DNA molecular size curve. Partial digestion products (*) have been excluded from the total molecular size values. Fragments with high molecular sizes such as ~21 kb Eco RI and ~17 kb Kpn I fragments, have been deduced previously and are included to provide a comprehensive overview.

Kpn I/Not I (kb)	Kpn I (kb)	Kpn I/Hind III (kb)	Hind III (kb)	Hind III/Eco RI (kb)	Eco RI (kb)	Xho I/Eco RI (kb)	Kpn I/Eco RI (kb)	Xba I/Kpn I (kb)	Xba I (kb)	Xba I/Eco RI (kb)	Xba I/Xho I (kb)	Xho I (kb)
17.05 9.10 8.00 7.40 1.60 1.44 1.40 0.30	17.10 17.05 8.80 1.60 1.40 0.30	17.05 10.20 8.80 7.00 1.60 1.40 0.30	36.20 10.20	21.00 10.30 8.80 4.20 2.00 0.24	21.00 10.30 8.80 7.40* 5.80* 5.50* 4.20 3.40* 2.28* 2.00 0.24	21.00 10.30 4.90 4.20 3.10 2.00 0.81 0.24	17.05 10.30 6.40 4.20 2.50 1.95 1.63 1.40 0.48 0.30 0.24	17.05 9.10 6.40 2.60 2.60 2.20 1.65 1.43 1.40 0.81 0.37 0.30	20.00 9.10 6.40 3.20 2.60 2.60 2.20 0.33	20.00 9.10 5.40 3.20 2.20 1.83 1.22 1.15 0.90 0.66 0.33 0.20	20.00 9.10 4.65 2.95 2.60 2.60 2.20 1.80 0.33	21.80 19.70 4.90
46.29	46.25	46.35	46.40	46.54	46.54	46.55	46.45	45.91	46.43	46.19	46.23	46.40

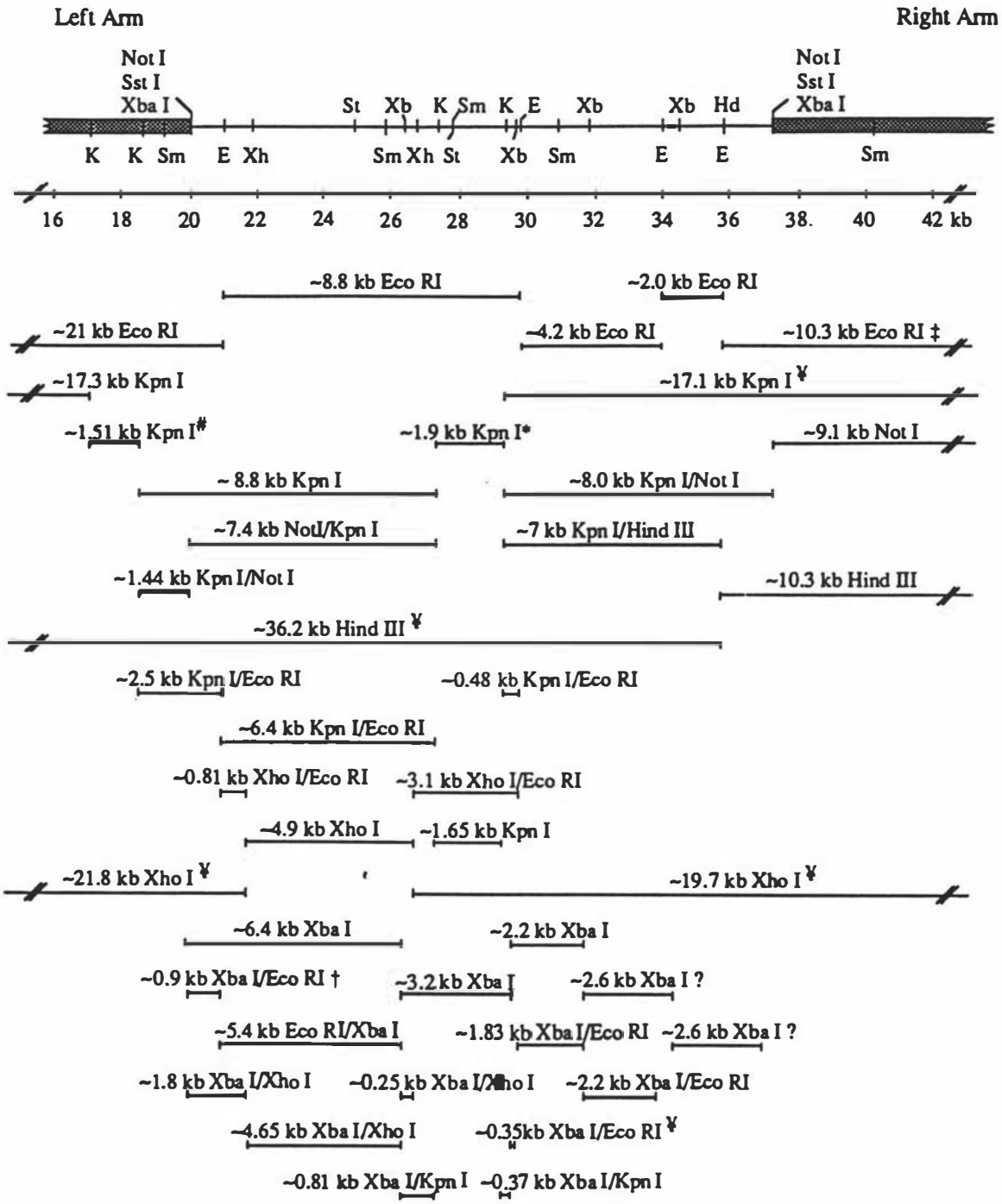


Figure 20: Position of fragments produced by the digestion of λOHNZ1 with a variety of restriction endonucleases or combinations of restriction enzymes

Observed fragments where the precise location has not been defined (?). Fragment sizes determined by deduction from other digest products using a total size of ~46.4 kb (¥). Partial digest product (*). The fragment (#) appeared to migrate as a ~1.4 kb fragment, however, data from figure 9 indicates that this fragment should have a size of ~1.51 kb. The ~10.3 kb Eco RI fragment (†) is described in Appendix 3 as a ~10.8 kb fragment which migrates anomalously.

K=Kpn I, Sm=Sma I, E=Eco RI, Xh=Xho I, St=Sst I, Xb=Xba I, Hd=Hind III

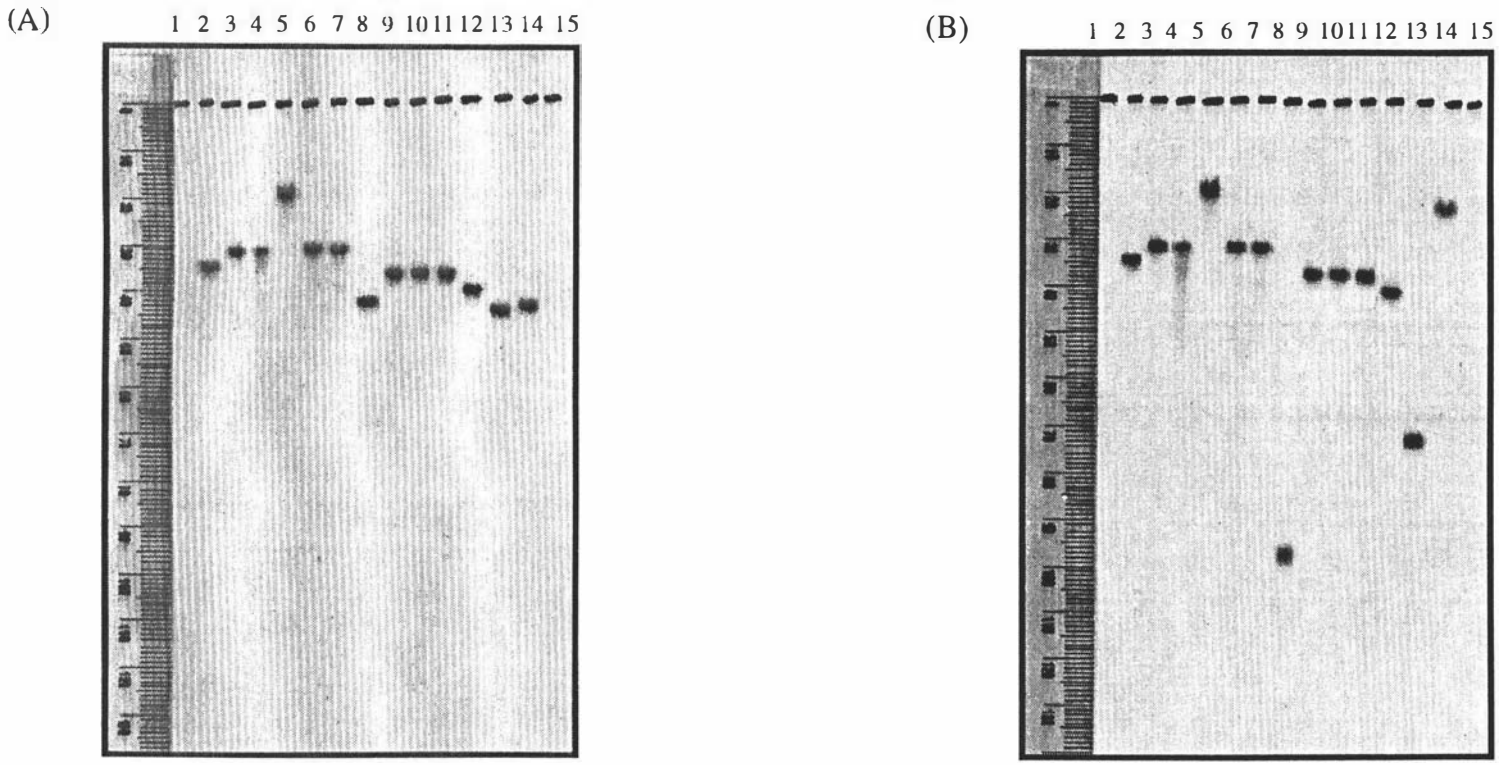


Figure 21: Southern hybridisation of digested λOHNZ1 DNA

(A). Digested fragments (Figure 19) were transferred to nylon GeneScreen plus membrane and hybridised with a radiolabelled probe derived from exon I sequences. The washed membrane (1x SSC, 68°C) was exposed to X-ray film.

(B). The bound exon I probe (A) was stripped from the membrane and exposed to X-ray film to checked for residual hybridisation signals. An exon II radiolabelled probe was hybridised to the stripped blot, washed under high stringency and autoradiographed.

(1) and (15) BRL 1 kb DNA ladder. λOHNZ1 DNA (~2 µg) digested with: (2) Kpn I/Not I; (3) Kpn I; (4) Kpn I/Hind III; (5) Hind III; (6) Hind III/Eco RI; (7) Eco RI; (8) Xho I/Eco RI; (9) Kpn I/Eco RI; (10) Xba I/Kpn I; (11) Xba I; (12) Xba I/Eco RI; (13) Xba I/Xho I; (14) Xho I.

Table 11: Fragments of λOHNZ1 (Figure 21) which hybridise to exon I and exon II

Restriction endonuclease	λOHNZ1 hybridisation fragments (kb)	
	Exon I	Exon II
Kpn I/Not I	~7.4	~7.4
Kpn I	~8.8	~8.8
Kpn I/Hind III	~8.8	~8.8
Hind III	~36.2	~36.2
Hind III/Eco RI	~8.8	~8.8
Eco RI	~8.8	~8.8
Xho I/Eco RI	~4.9	~0.81
Kpn I/Eco RI	~6.4	~6.4
Xba I/Kpn I	~6.4	~6.4
Xba I	~6.4	~6.4
Xba I/Eco RI	~5.4	~5.4
Xba I/Xho I	~4.65	~1.8
Xho I	~4.9	~21.8

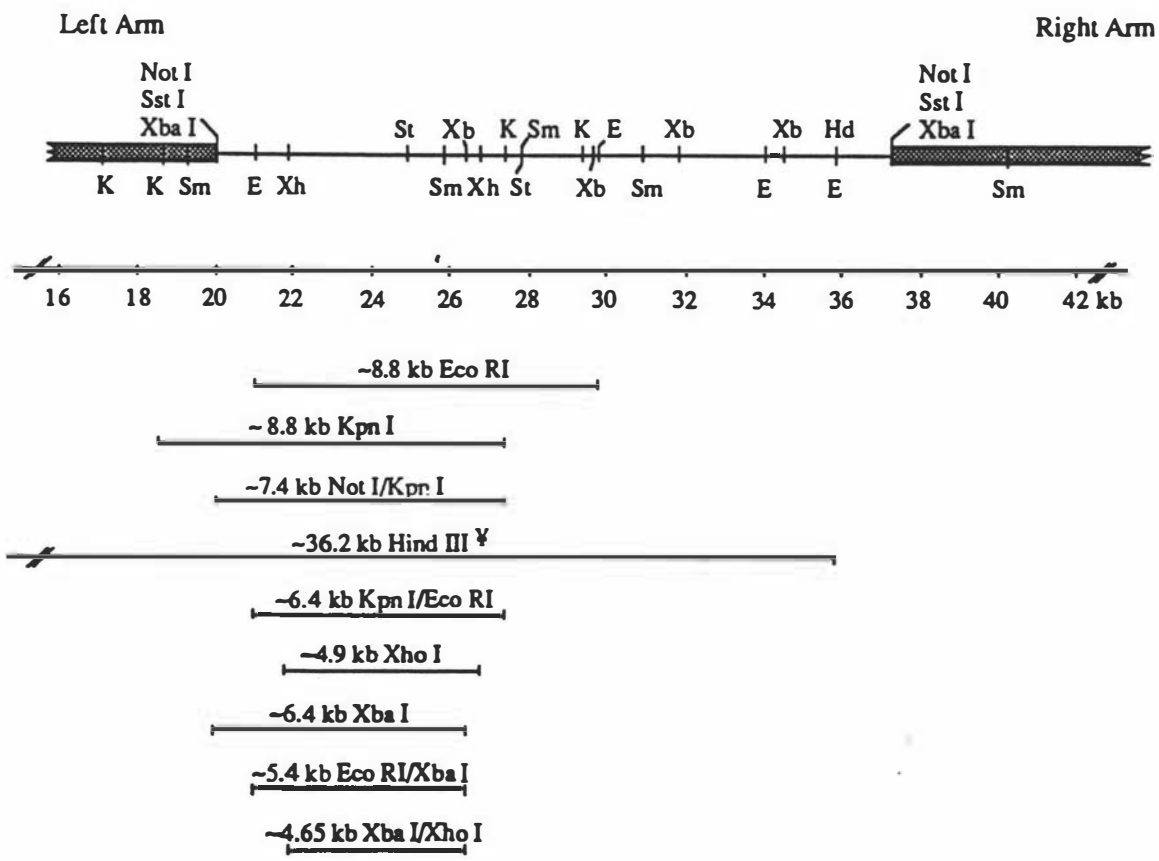


Figure 22: Pictorial display of restriction fragments (Figure 19) which hybridised to an exon I radiolabelled probe

¥ indicates that the size of this fragment was calculated from the total molecular size values and not deduced from the DNA standard curve.

K=Kpn I, Sm=Sma I, E=Eco RI, Xh=Xho I, St=Sst I, Xb=Xba I, Hd=Hind III

Figure 23: Schematic diagram of digest fragments which hybridised to a probe representing exon II sequences of bovine lactoferrin

Fragment sizes deduced from the total size values (¥).

K=Kpn I, Sm=Sma I, E=Eco RI, Xh=Xho I, St=Sst I, Xb=Xba I, Hd=Hind III

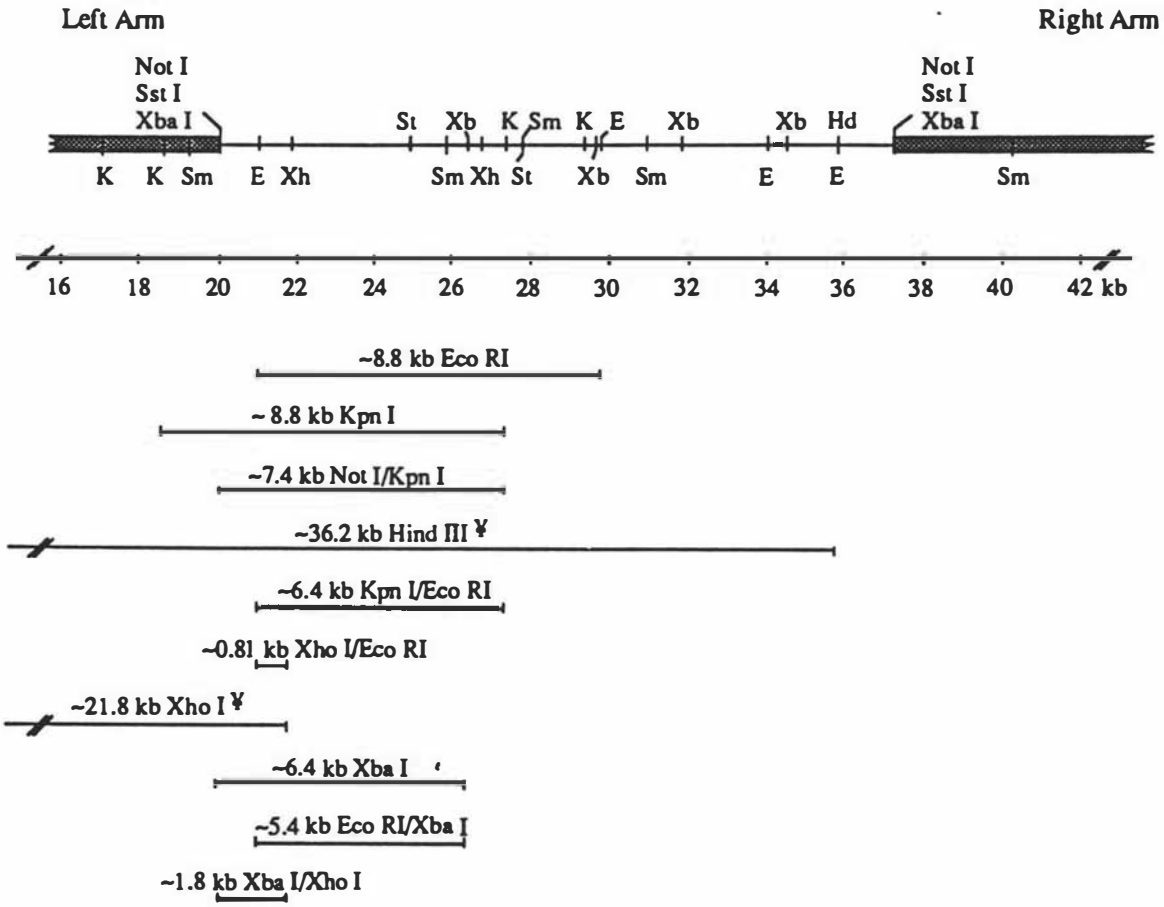


Figure 24: Restriction analysis of λOHNZ1

λOHNZ1 DNA (~2 μg) was digested for one hour at 37°C with the restriction endonucleases listed below. The samples were separated by electrophoresis in a 0.7% agarose gel in 1x TBE at ~70 V for 3-4 hours. The gel was stained in ethidium bromide and the fragments visualised and photographed under UV illumination.

Lane 1.	BRL 1 kb DNA ladder
Lane 2.	λOHNZ1 DNA (Uncut)
Lane 3.	λOHNZ1 DNA (~2 μg) digested with (Eco RI/Xho I)
Lane 4.	" " " " " (Eco RI)
Lane 5.	" " " " " (Sst I/Eco RI)
Lane 6.	" " " " " (Sst I)
Lane 7.	" " " " " (Kpn I/Sst I)
Lane 8.	" " " " " (Xho I/Kpn I/Sst I)
Lane 9.	" " " " " (Xho I/Kpn I/Eco RI)
Lane 10.	" " " " " (Xho I/Kpn I)
Lane 11.	" " " " " (Eco RI/Kpn I)
Lane 12.	" " " " " (Kpn I)
Lane 13.	" " " " " (Xho I/Sst I)
Lane 14.	" " " " " (Xho I)
Lane 15.	BRL 1 kb DNA ladder

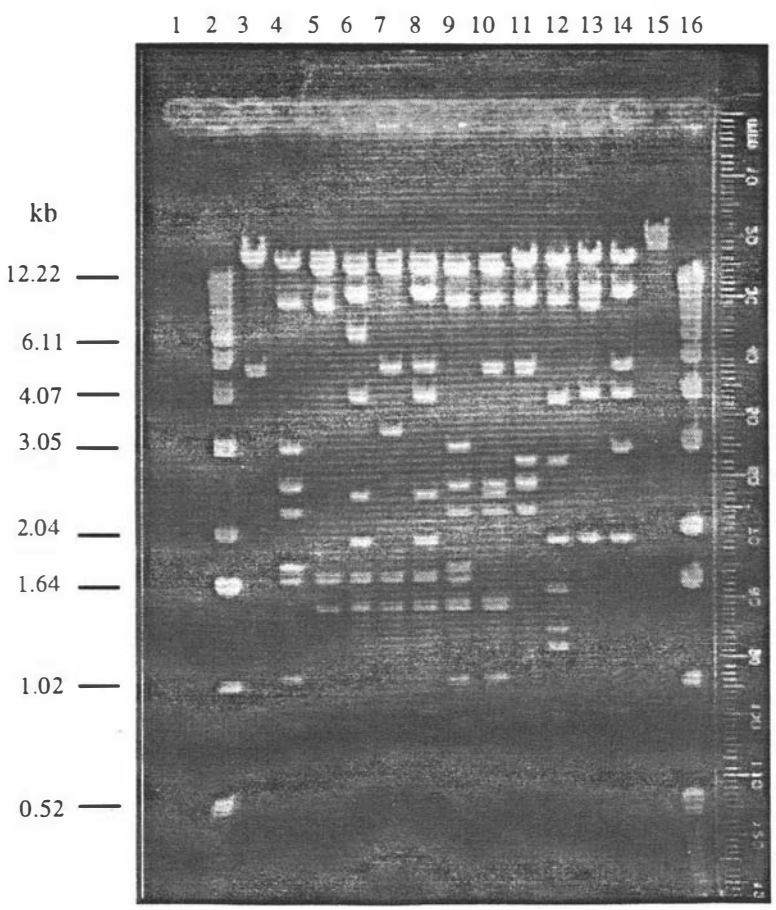


Table 12: Molecular sizes of restriction fragments shown in figure 24

Partial digestion products (*) have been excluded from the total molecular size values. All molecular values are approximate and deduced from a DNA standard molecular size curve.

Eco RI/Xho I (kb)	Eco RI (kb)	Sst I/Eco RI (kb)	Sst I (kb)	Kpn I/Sst I (kb)	Xho I/Kpn I/Sst I (kb)	Xho I/Kpn I/Eco RI (kb)	Xho I/Kpn I (kb)	Eco RI/Kpn I (kb)	Kpn I (kb)	Xho I/Sst I (kb)	Xho I (kb)
21.00 10.30 4.90 4.20 3.15 2.00 0.86 0.20	21.00 10.30 8.80 4.20 2.00 0.20	20.00 9.10 4.00 2.90 1.95 1.58 1.32 1.23 0.86 0.86 0.72 0.50 0.20	20.00 9.10 4.90 2.90 2.65 2.65 2.25 0.86 0.75	17.05 9.10 4.90 2.65 2.50 2.20 1.55 1.45 1.45 1.05 0.86 0.74 0.30 0.10	17.05 9.10 3.15 2.65 2.25 1.85 1.65 1.55 1.45 1.45 1.05 0.83 0.70 0.45 0.20	17.05 10.30 8.80* 4.90 4.15 2.45 2.15* 1.95 1.85* 1.65 1.45 0.89 0.70 0.51 0.20 0.20	17.10 17.05 4.90 3.40 2.67* 1.85* 1.65 1.45 0.89* 0.72 0.20	17.05 10.30 6.40 4.20 2.45 1.95 1.65 1.45 0.48 0.20	17.10 17.05 11.20* 8.80 1.85* 1.65 1.45 0.20	20.00 9.10 4.90* 3.15 2.60 2.60 2.25 1.85 1.65 1.05 0.83 0.69 0.42	21.80 19.80 4.80
46.61	46.50	45.22	46.06	45.90	45.38	46.40	46.47	46.13	46.25	46.19	46.40

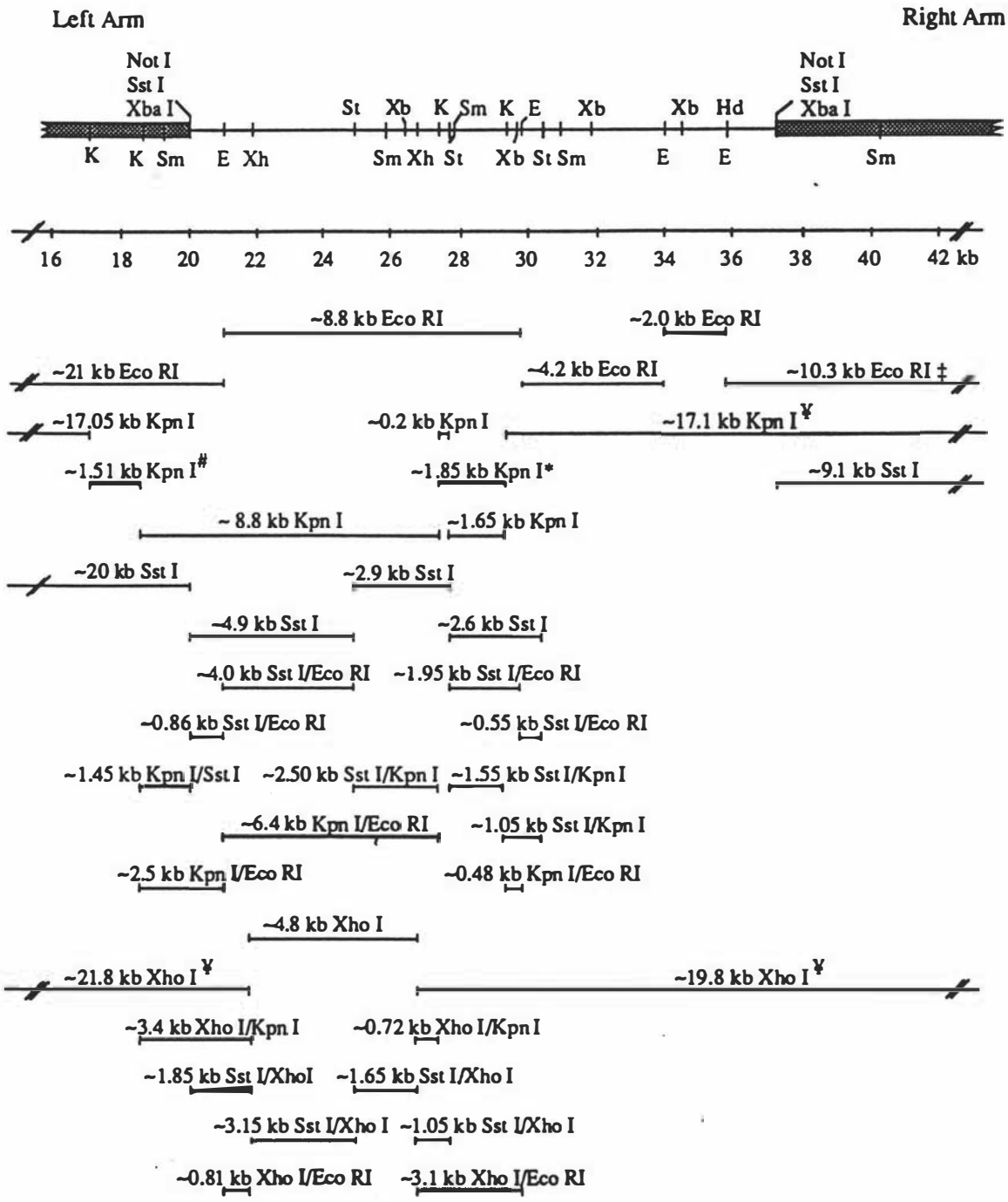
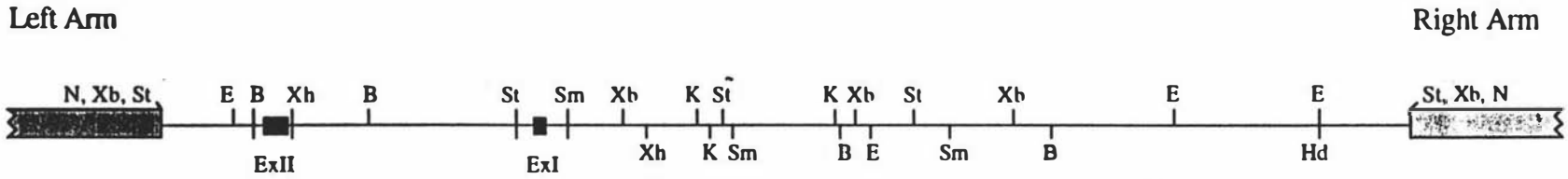


Figure 25: Schematic representation of digestion fragments of λOHNZ1

The previously discussed anomalous ~10.8 kb Eco RI fragment (†) and ~1.51 kb Kpn I fragment (#) are indicated. Partial fragments (*) and fragment sizes which were deduced from the total fragment sizes of other digestions (¥) are also shown on the map.

K= Kpn I, E=Eco RI, Sm=Sma I, Xh=Xho I, St=Sst I, Hd=Hind III, Xb=Xba I

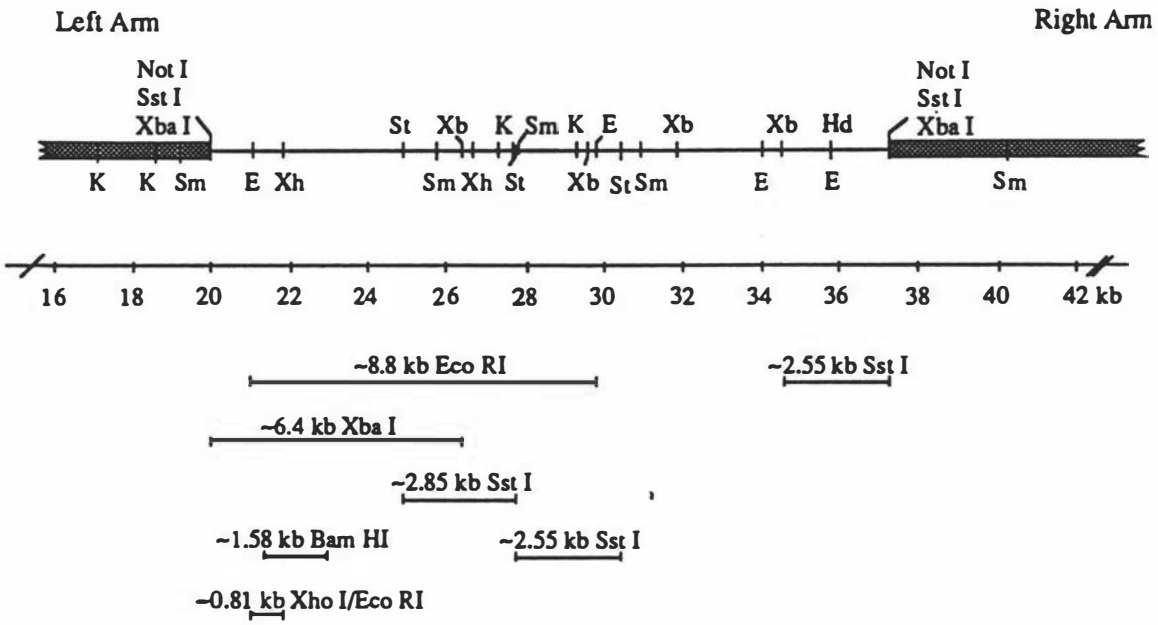
Figure 26: Summary restriction map of λOHNZ1



N=NotI St=SstI Xb=XbaI E=EcoRI B=BamHI Xh=XhoI Sm=SmaI K=KpnI Hd=HindIII

Exon I and exon II are located within the boundaries of the restriction sites shown.

Figure 27: Schematic representation of fragments analysed during this study



3.5 Characterisation of Subcloned Fragments

3.5.1 Restriction Analysis of ~6.4 kb Xba I Fragment

A probe derived from exon II sequences was shown to hybridise to a ~8.8 kb Eco RI fragment and a ~1.58 kb Bam HI fragment of the λOHNZ1 clone isolated from the Stratgene library (Section 3.4). Hybridisation of the same probe to isolated fragments from clone I, derived from a Clonetech library, indicated that a 2.025 kb Eco RI fragment and a 0.531 kb Bam HI fragment bind the probe (Figures 5 and 6). This discrepancy was investigated by restriction analysis of a ~6.4 kb Xba I fragment, isolated from λOHNZ1, which had previously been shown to contain exon I and exon II sequences (Section 3.4).

The ~6.4 kb Xba I fragment was subcloned into the pBluescript™ KS+ and the orientation was determined by Eco RI digestion as shown in figure 28. Clones with the orientation shown in figure 28A were chosen for further investigation.

DNA from isolate A was digested with several restriction endonucleases. Fragment sizes were predicted from the restriction map of λOHNZ1 (Figure 26). These values are shown in table 13. All the digestions gave the fragmentation profiles expected (Figure 29).

Bam HI digestion yielded five fragments of ~4.2, 2.25, 1.58, 0.86 and 0.64 kb. The ~0.86 kb fragment was not observed in the Bam HI digestion of λOHNZ1 (Figure 13A) suggesting that Xba I cleaves between two Bam HI restriction sites in λOHNZ1. This positioned a Bam HI restriction site ~0.86 kb from the 5' end of the Xba I insert in isolate A (Figure 29). The ~2.25, 1.58 and 0.64 kb Bam HI fragments were common to the Bam HI digestions of both λOHNZ1 and isolate A. Earlier hybridisation analyses of λOHNZ1 had shown that exon I and exon II sequences of bovine lactoferrin were contained within a ~0.64 kb and a ~1.58 kb Bam HI fragment respectively (Figure 10 and 13). The position of the ~0.64 kb Bam HI fragment within λOHNZ1 could not be established. The ~1.58 kb fragment was mapped ~1.28 kb from the left arm within λOHNZ1 (Figure 27). This ~1.28 kb plus the vector (~3.0 kb) explained the observed ~4.2 kb fragment in the Bam HI digestion of isolate A. Based upon the relative position of λOHNZ1 digest fragments which hybridised to exon I or exon II sequences, it was concluded that the ~0.64 kb and ~1.58 kb Bam HI fragments were not adjacent within the clone (Figures 22 and 23). Therefore, the ~2.25 kb Bam HI fragment was positioned between these two fragments within isolate A.

The ~1.1 kb fragment visible in the Sst I/Bam HI digest (Figure 29) placed a Bam HI restriction site ~1.1 kb from the cloning junction (Figure 30). The individual

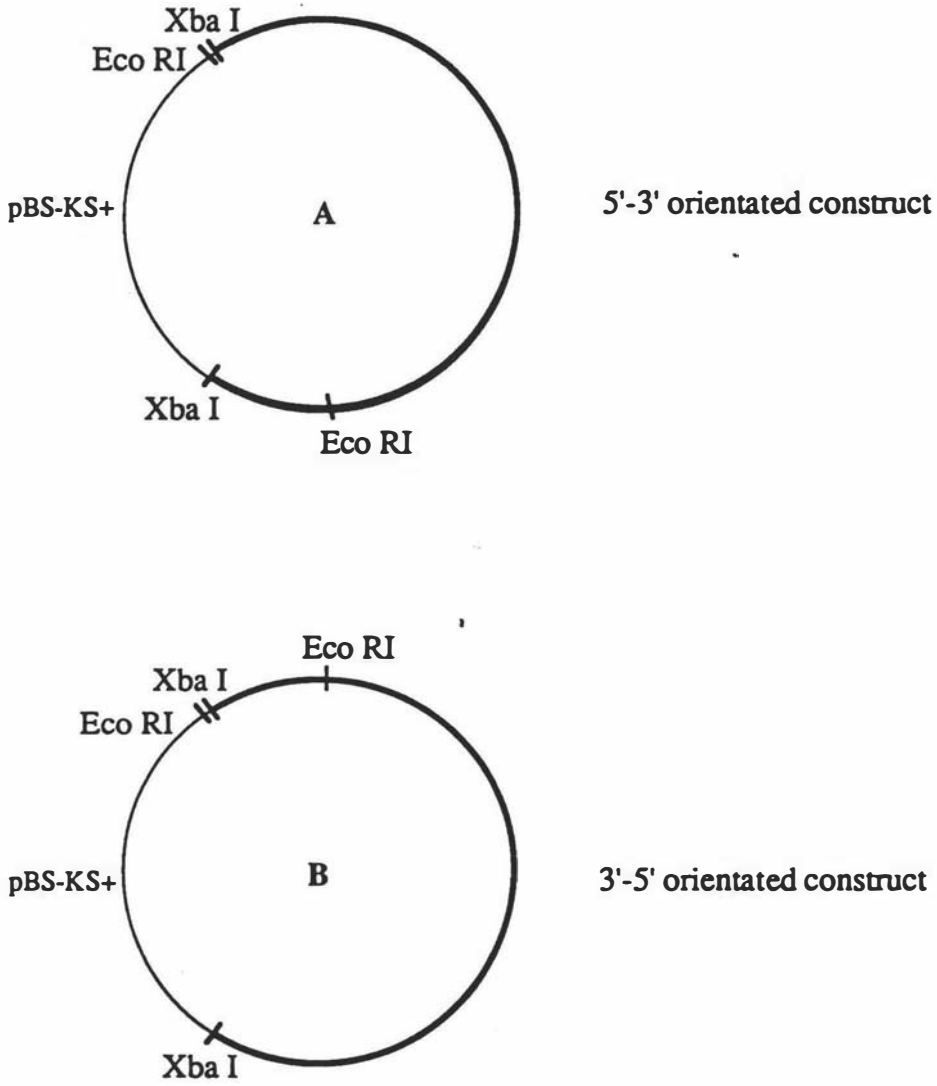


Figure 28: Orientation of the 6.4 kb Xba I fragment in pBluescript®

Bluescript constructs containing the ~6.4 kb insert were digested with the restriction endonuclease Eco RI. Fragments with sizes of ~5.4 kb and ~3.85 kb indicated a construct oriented 5'-3' with respect to the lactoferrin transcription start site (Isolate A). Eco RI fragments of ~8.3 kb and ~0.96 kb were diagnostic of a 3'-5' oriented insert (Isolate B).

Sst I and Xho I digests gave a single broad band on the gel. In each digest this represents two fragments. The presence of a unique ~0.3 kb fragment in the Eco RI/Bam HI digest implied that Eco RI cleaved ~0.3 kb from a Bam HI restriction site. The predicted ~0.68 kb and ~0.6 kb fragments in the Sma I/Bam HI digestion appeared as a broad band indicating that these fragments had not been separated by the gel electrophoresis. Consequently these fragments had a slightly different mobility than fragments of the expected molecular sizes. These results are illustrated in figure 30.

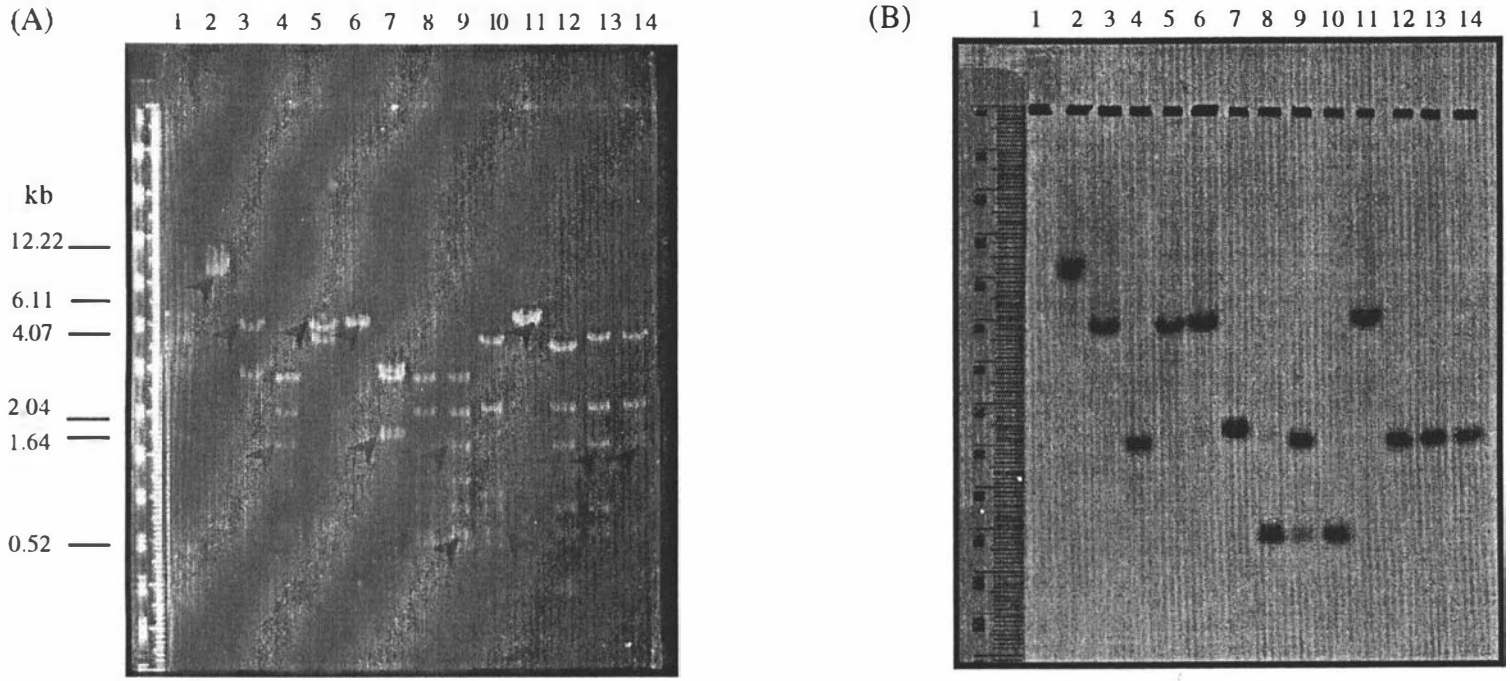


Figure 29: Restriction digests and Southern blot of Isolate A

(A). Plasmid DNA (~2 µg) was digested for one hour at 37°C with the restriction endonucleases listed below. The samples were separated by electrophoresis in a 0.7% agarose gel in 1x TAE for ~3 hours at 80 V. The fragments were stained with ethidium bromide and photographed under UV illumination. Arrowheads indicate the fragments which hybridised to the probe.

(B). Digest fragments (A) were transferred to a nylon membrane and hybridised at 68°C with a radiolabelled probe comprising of exon II sequences. The membrane was washed at high stringency (1x SSC, 68°C) and exposed to X-ray film at room temperature for ~45 minutes.

(1) BRL 1 kb ladder. Isolate A DNA (~2 µg) digested with: (2) Sma I; (3) Sma I/Sst I; (4) Sma I/Sst I/Bam HI; (5) Sma I/Xho I; (6) Sst I; (7) Sst I/Xho I; (8) Sst I/Xho I/Bam HI; (9) Sst I/Bam HI; (10) Xho I/Bam HI; (11) Xho I; (12) Eco RI/Bam HI; (13) Bam HI; (14) Sma I/Bam HI.

Table 13: Restriction fragments of Isolate A

Digestion fragment sizes were predicted from the restriction map of λOHNZ1 (Figure 26).

Restriction Endonuclease	Predicted Fragment Sizes (kb)	Observed Fragment Sizes (kb)	Total Molecular Size (kb)
Sma I	8.66, 0.6	~9.0, ~0.67	9.67
Sma I/Sst I	4.9, 2.96, 0.9, 0.6	~4.85, ~2.9, ~0.86, ~0.67	9.28
Sma I/Sst I/Bam HI	2.96, 2.1, 1.55, 1.28, 0.68, 0.6, 0.15	~2.8, ~2.1, ~1.58, ~1.07, ~0.67, ~0.62, ~0.2	9.04
Sma I/Xho I	4.8, 4.0, 0.6	~4.7, ~4.05, ~0.67	9.42
Sst I	4.9, 4.46	~4.8, ~4.8	9.60
Sst I/Xho I	3.1, 2.96, 1.8, 1.6	~3.05, ~2.9, ~1.7, ~1.6	9.25
Sst I/Xho I/Bam HI	2.96, 2.1, 1.28, 1.03, 0.86, 0.68, 0.54, 0.15	~2.9, ~2.1, ~1.07, ~0.98, ~0.82, ~0.62, ~0.54	9.03
Sst I/Bam HI	2.96, 2.07, 1.55, 1.28, 0.86, 0.68, 0.15, 0.1	~2.9, ~2.1, ~1.58, ~1.1, ~0.82, ~0.62, ~0.2	9.32
Xho I/Bam HI	4.3, 2.2, 1.03, 0.86, 0.68, 0.52	~4.2, ~2.18, ~0.98, ~0.81, ~0.62, ~0.54	9.33
Xho I	4.8, 4.6	~4.7, ~4.7	9.40
Eco RI/Bam HI	3.9, 2.2, 1.55, 0.86, 0.68, 0.3	~3.9, ~2.2, ~1.58, ~0.86, ~0.64, ~0.3	9.48
Bam HI	4.3, 2.2, 1.55, 0.86, 0.68	~4.2, ~2.25, ~1.58, ~0.86, ~0.64	9.53
Sma I/Bam HI	4.3, 2.2, 1.55, 0.68, 0.6, 0.19	~4.2, ~2.3, ~1.58, ~0.73, ~0.67	9.48

Following Southern transfer to a nylon membrane, the fragments were hybridised with a probe derived from exon II sequences (Figure 29B). Specific hybridisation signals were obtained for all digestions (Table 14). Two hybridisation signals were visible within the Sst I/Bam HI digestion. The ~1.55 kb band represented a fragment which was predicted to hybridise. Weak hybridisation of a ~0.54 kb fragment suggested that this digest had been contaminated, as a fragment of this size hybridised within both of the adjacent digestions.

These results showed the specific interaction of the exon II probe with Isolate A DNA and confirmed that exon II was contained within a ~1.58 kb Bam HI fragment. This data defined the position of the Bam HI restriction sites within this fragment and allowed the location of the ~0.64 kb Bam HI fragment, known to contain exon I, to be specified. The location of exon I within isolate A was confirmed by the identification of additional restriction fragment which hybridised to a probe derived from exon I sequences of bovine lactoferrin (Figure 31A). The digestion products obtained are listed in table 15. Fragments of the expected size were obtained for all digests except for those within the Sma I/Xho I profile.

Figure 30: Schematic representation of fragments from restriction digestions of isolate A

A predicted ~0.19 kb Sma I/Bam HI fragment (?) could not be detected on the gel shown in figure 29.

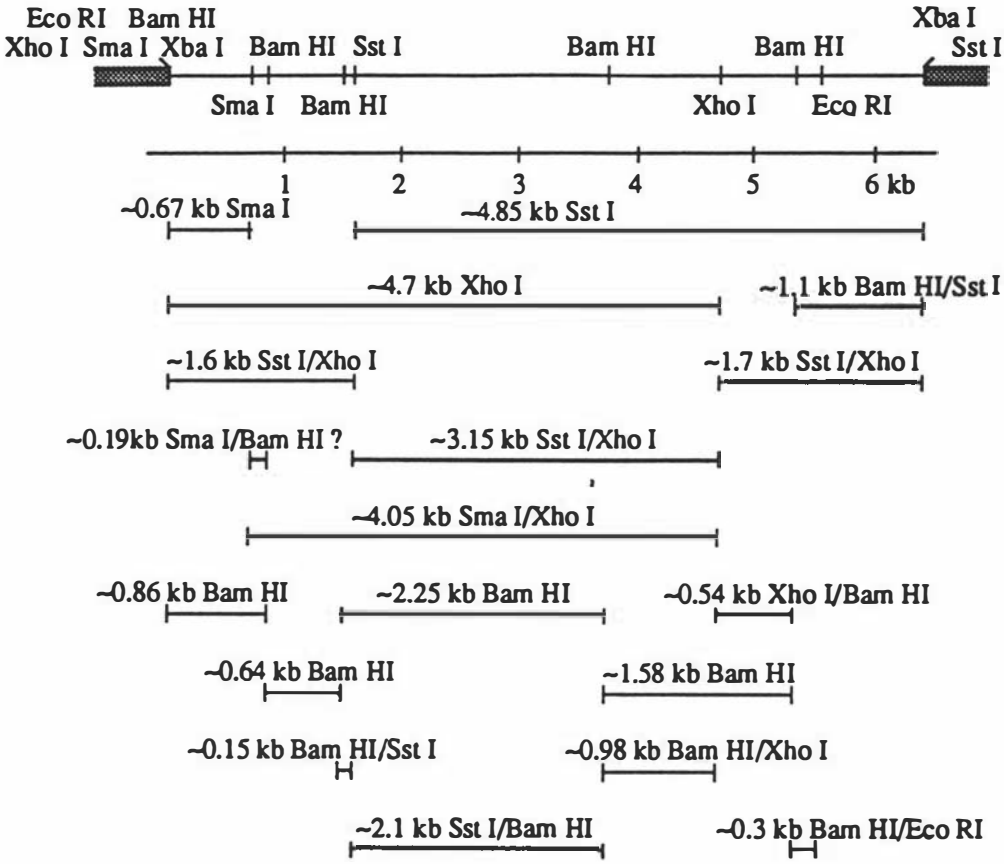


Table 14: Fragments of Isolate A which hybridised to exon II

A suspected contaminating fragment (◊) is indicated.

Restriction Endonuclease	Observed Hybridisation Fragment Size (kb)
Sma I	~9.0
Sma I/Sst I	~4.85
Sma I/Sst I/Bam HI	~1.58
Sma I/Xho I	~4.7
Sst I	~4.8
Sst I/Xho I	~1.7
Sst I/Xho I/Bam HI	~0.54
Sst I/Bam HI	~1.58, ~0.540
Xho I/Bam HI	~0.54
Xho I	~4.7
Eco RI/Bam HI	~1.58
Bam HI	~1.58
Sma I/Bam HI	~1.58

Table 15: Restriction fragments of Isolate A

Partial digestion fragments (*) have been excluded from the total molecular size values.

Restriction Endonuclease	Predicted Fragment Sizes (kb)	Observed Fragment Sizes (kb)	Total Molecular Size (kb)
Xba I	6.4, 2.96	~6.4, ~3.0	9.4
Eco RI	5.4, 3.9	~5.4, ~3.9	9.3
Sst I/Xho I	3.1, 2.96, 1.8, 1.5	~3.05, ~2.85, ~1.75, ~1.65	9.3
Sst I/Bam HI	2.96, 2.07, 1.55, 1.28, 0.86, 0.68, 0.15	~2.85, ~2.1, ~1.58, ~1.1, ~0.82, ~0.62	9.07
Sst I	4.9, 4.46	~4.8, ~4.8	9.6
Sst I/Sma I	4.9, 2.96, 0.9, 0.6	~4.8, ~2.95, ~0.86, ~0.65	9.26
Sma I	8.66, 0.6	~9.16, ~0.65	9.81
Sma I/Xho I	4.8, 4.0, 0.6	~4.8, ~4.0, ~3.2, ~2.95, ~1.9*, ~1.8, ~0.82*, ~0.65	17.4
Sma I/Bam HI	4.3, 2.2, 1.55, 0.68, 0.6, 0.1	~4.3, ~2.2, ~1.58, ~0.65, ~0.65	9.38
Bam HI	4.3, 2.2, 1.55, 0.86, 0.68	~4.3, ~2.25, ~1.58, ~0.82, ~0.62	9.57
Bam HI/Xho I	4.3, 2.2, 1.03, 0.86, 0.68, 0.52	~4.3, ~2.25, ~0.94, ~0.86, ~0.62, ~0.56	9.53
Xho I	4.8, 4.6	~4.9, ~4.9,	9.8

The Sma I/Xho I digestion produced several fragments of unknown origin. Collectively these fragments gave a total molecular size of 17.4 kb which was much larger than the other digestion totals. This implied that this digest was contaminated. The expected 1.8 kb and 1.5 kb Sst I/Xho I fragments were poorly resolved, appearing as ~1.75 kb and ~1.65 kb fragments. The ~9.16 kb Sma I fragment appeared as a broad overloaded band. Due to experimental limitations the size of this fragment could not be resolved accurately. These results are summarised in figure 32.

Specific signals were observed from the hybridisation of a probe derived from exon I (Figure 31B, Table 16). Two hybridisation signals were visible in the Sma I/Xho I digestion profile. The intense ~4.0 kb band was the fragment predicted to hybridise to the probe. The fainter ~0.82 kb band was the same size as the Sma I fragment which hybridised to the probe. The faint appearance of this fragment within the digestion suggested that this fragment represented a partial digestion product. The unusual digestion profile and two hybridisation fragments implied that the Sma I/Xho I digest was contaminated. For this reason, this digest could not be interpretated.

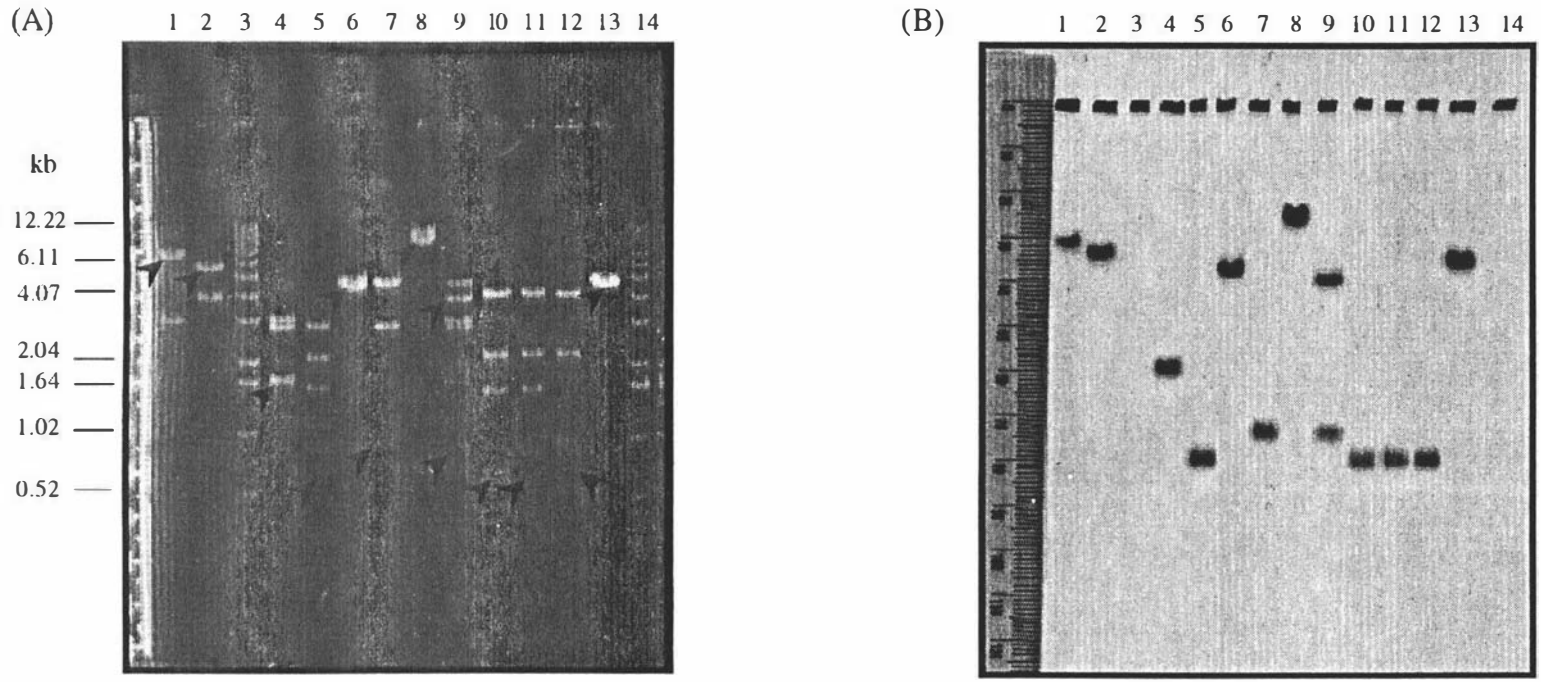


Figure 31: Restriction digests and Southern blot of Isolate A

(A). Plasmid DNA (~2 µg) was digested for one hour at 37°C with the restriction endonucleases listed below. The samples were separated by electrophoresis through a 0.8% agarose gel in 1x TAE for ~3 hours at 80 V. The fragments were stained with ethidium bromide and photographed under UV illumination. Arrowheads indicate fragments which hybridise to the probe.

(B). DNA fragments (A) were transferred to a nylon membrane and hybridised at 68°C with an exon I derived radiolabelled probe. After washing at high stringency (1x SSC, 68°C) the filter was exposed to X-ray film at room temperature for ~40 minutes.

Isolate A DNA (~2 µg) digested with: (1) Xba I; (2) Eco RI; (4) Sst I/Xho I; (5) Sst I/Bam HI; (6) Sst I; (7) Sst I/Sma I; (8) Sma I; (9) Sma I/Xho I; (10) Sma I/Bam HI; (11) Bam HI; (12) Bam HI/Xho I; (13) Xho I. (3) and (14) BRL 1 kb DNA ladder

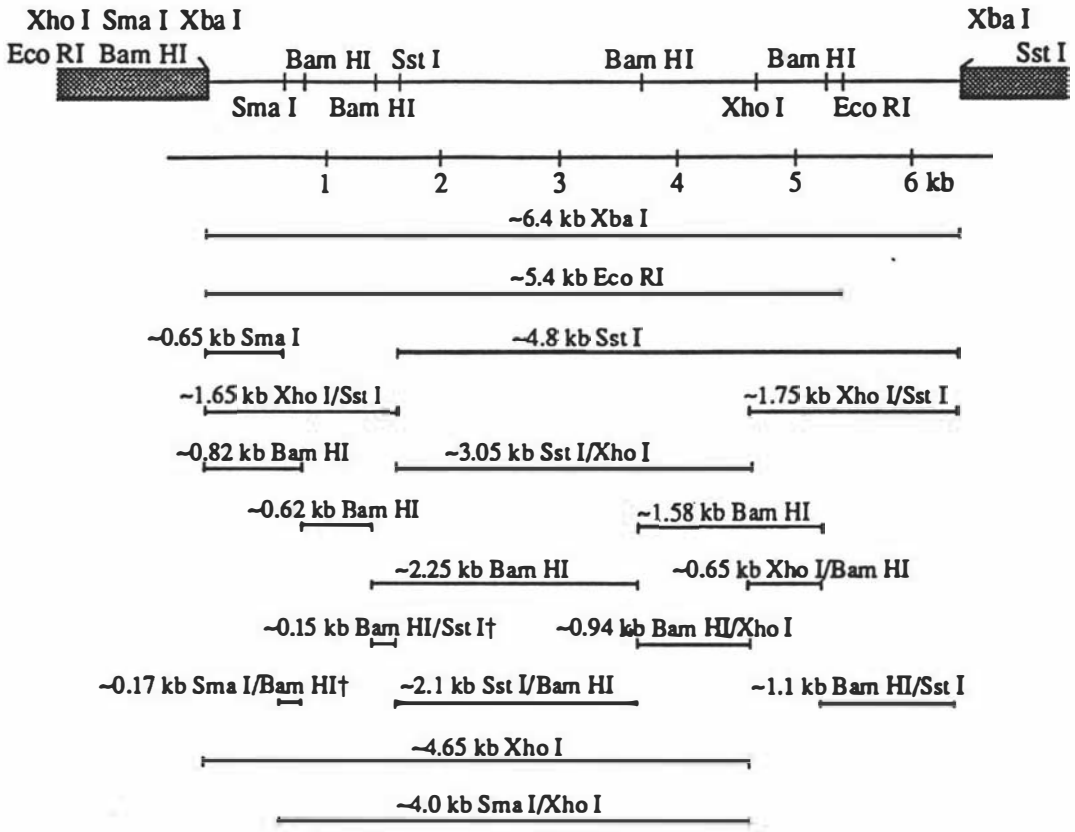


Figure 32: A partial restriction map of isolate A

After digesting Isolate A with restriction endonucleases, fragments were separated by gel electrophoresis as described in figure 31. A standard molecular size curve was constructed from the BRL 1 kb DNA ladder and used to deduce the sizes of the digest products. Predicted digest fragments (†) which were not observed are also shown.

Table 16: Hybridisation analysis of Isolate A

A ~0.82 kb Sma I/Xho I partial digestion product (*) is also shown.

Restriction Endonuclease	Observed Hybridisation Fragment Size (kb)
Xba I	~6.4
Eco RI	~5.4
Sst I/Xho I	~1.65
Sst I/Bam HI	~0.62
Sst I	~4.8
Sst I/Sma I	~0.86
Sma I	~9.16
Sma I/Xho I	~4.0, ~0.82*
Sma I/Bam HI	~0.65
Bam HI	~0.62
Bam HI/Xho I	~0.62
Xho I	~4.9

Collectively, the digestion profiles of Isolate A and the hybridisation results using probes from both exon I and exon II confirmed the position of the ~6.4 kb Xba I fragment within this subclone. The cleavage sites of six restriction endonucleases were defined and agreed with values predicted from the earlier analysis of λOHNZ1 (Figure 26). A Bam HI fragment of ~1.58 kb from both isolate A and λOHNZ1 hybridised to a probe representing exon II. In contrast, a 531 bp Bam HI fragment from clone I (Clonetech) was shown to contain exon II. This discrepancy is discussed in section 3.6.

3.5.2 Analysis of ~2.55 kb Sst I λOHNZ1 fragments

Restriction analysis had shown previously that two ~2.65 kb fragments were produced by Sst I cleavage of λOHNZ1 (Figure 24). Both of these fragments contained restriction sites for Eco RI (Figure 16A). One of these fragments had been mapped to the centre of the λOHNZ1 clone, but the location of the other ~2.65 kb fragment could not be defined due to lack of experimental data.

Three subclones containing a ~2.55 kb insert had been isolated during the subcloning of the ~2.8 kb Sst I fragment from λOHNZ1 which contains the bovine lactoferrin promoter. Each of these recombinant vectors (Isolates C, D, and E), were ~5.5 kb in size and were characterised by cleavage with several restriction endonucleases known to be diagnostic for λOHNZ1 (Figure 33). The digestion fragments obtained are shown in table 17.

Restriction mapping analysis of the λOHNZ1 clone had identified a ~0.20 kb Eco RI fragment (Table 12, Appendix 3). This fragment was observed in the Eco RI digestions of isolates C and D suggesting that the ~0.20 kb Eco RI fragment was located within the centre of the λOHNZ1 clone. This information was combined with previous data (Appendix 3) and restriction maps of isolates C and D were produced using the same strategy as previously described (Figures 34 and 35). Isolate C and D contained the same insert cloned into the Bluescript® vector in different orientations.

(1). BRL 1 kb DNA ladder
(2). Isolate C digested with (Sst I)
(3). Isolate C " " (Eco RI)
(4). Isolate C " " (Kpn I)
(5). Isolate C " " (Bam III)
(6). Isolate C " " (Hind III)
(7). Isolate C " " (Sma I)
(8). Isolate D " " (Sst I)
(9). Isolate D " " (Eco RI)
(10) Isolate D " " (Kpn I)
(11). Isolate D " " (Bam III)
(12). Isolate D " " (Hind III)
(13). Isolate D " " (Sma I)
(14). Isolate E " " (Sst I)
(15). Isolate E " " (Eco RI)
(16). Isolate E " " (Kpn I)
(17). Isolate E " " (Bam III)
(18). Isolate E " " (Hind III)
(19). Isolate E " " (Sma I)
(20). BRL 1 kb DNA ladder

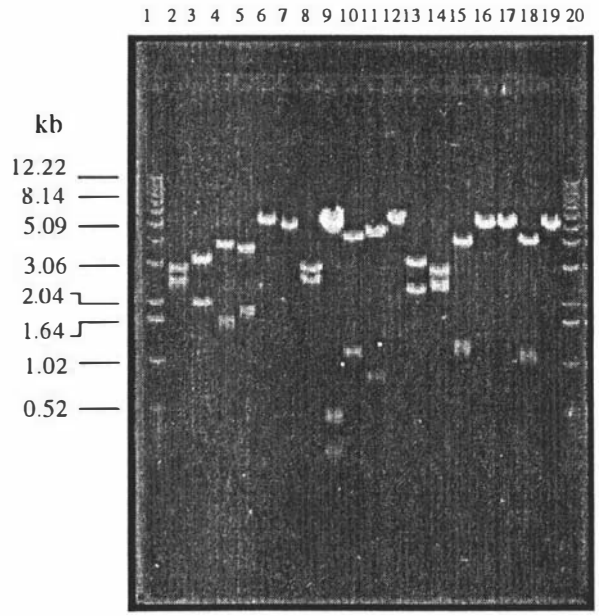


Figure 33: Restriction analysis of Sst I subclones

DNA (~1.5 µg) from three λOHNZ1 subclones was digested at 37°C for one hour in the appropriate buffer and the fragments were separated by gel electrophoresis through a 0.8% agarose gel in 1x TAE buffer. The fragments were stained with ethidium bromide and photographed with the aid of a UV transilluminator.

Table 17: Restriction analysis of Isolates C, D and E

Isolate	Restriction endonuclease	Observed fragments molecular size (kb)	Total molecular size (kb)
C	Sst I	~2.9, ~2.55	5.45
C	Eco RI	~3.3, ~2.04, ~0.20	5.54
C	Kpn I	~4.05, ~1.52	5.57
C	Bam HI	~3.7, ~1.8	5.5
C	Hind III	~5.5	5.5
C	Sma I	~5.15, ~0.3	5.45
D	Sst I	~2.9, ~2.58	5.48
D	Eco RI	~4.9, ~0.42, ~0.20	5.52
D	Kpn I	~4.3, ~1.25	5.55
D	Bam HI	~4.5, ~0.79	5.29
D	Hind III	~5.5	5.5
D	Sma I	~3.05, ~2.25	5.30
E	Sst I	~2.9, ~2.55	5.45
E	Eco RI	~4.3, ~1.25	5.55
E	Kpn I	~5.5	5.5
E	Bam HI	~5.5	5.5
E	Hind III	~4.3, ~1.14	5.44
E	Sma I	~5.5	5.5

Figure 34: Schematic representation of digestion fragments of isolate C

Sm=Sma I, K=Kpn I, B=Bam HI, E=Eco RI

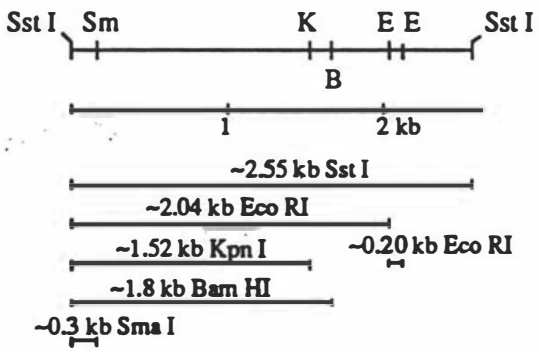
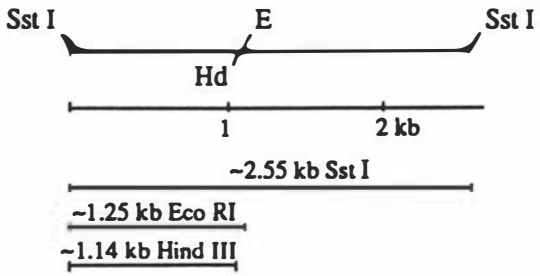


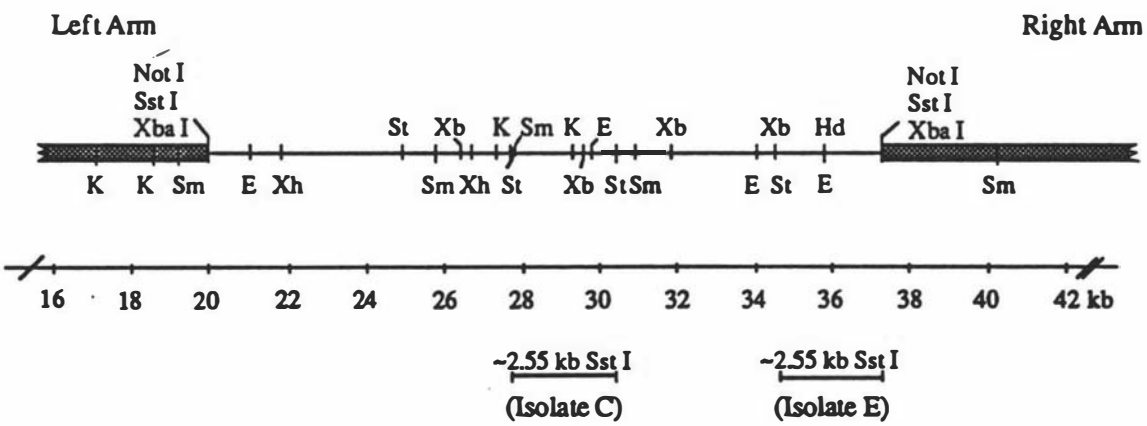
Figure 35: Schematic representation of restriction digestion fragments from isolate E

Hd=Hind III, E=Eco RI



The insert within isolate C and D represented the ~2.65 kb Sst I fragment which had been mapped previously to the centre of the λOHNZ1 clone (Section 3.4). The presence of a Hind III restriction site within isolate E (Figure 33) implied that this ~2.55 kb Sst I fragment was located next to the right arm within the λOHNZ1 clone (Figure 36). The absence of a Bam HI, Kpn I and Sma I restiction sites within isolate E supported this proposal (Figure 36). Collectively, these data were used to locate the insert in isolate E adjacent to the right arm of the λOHNZ1 clone (Figure 36). Figure 36 summarises the location of the ~2.55 kb Sst I fragments which were contained within isolates C, D and E.

Figure 36: Schematic representation showing the location of the ~2.55 kb Sst I fragments within λOHNZ1



Specific hybridisation signals were obtained for ~8.8 kb Eco RI and ~2.85 kb Sst I restriction fragments of λOHNZ1 when probed with exon I of bovine lactoferrin (Figure 27). As the primary focus of this study was to isolate and characterise the promoter region of the bovine lactoferrin gene, these fragments were subcloned from λOHNZ1 into the plasmid vector Bluescript® KS+ and characterised by restriction analysis. The strategy used is discussed in the following sections.

3.5.3 Restriction Analysis of the ~8.8 kb Eco RI Fragment

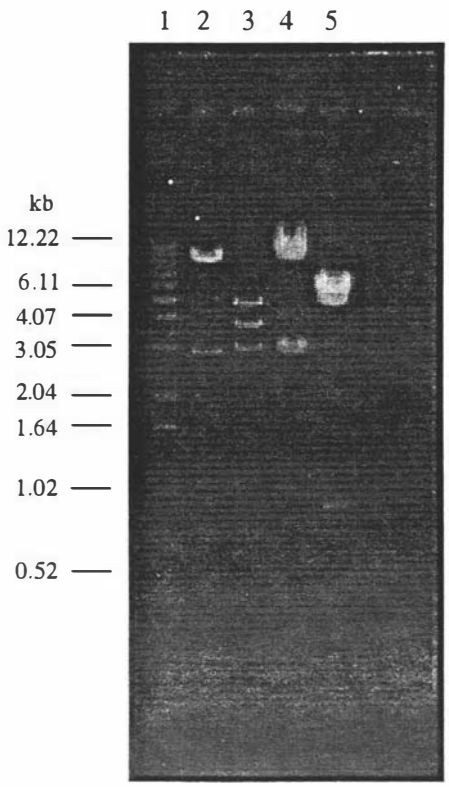
The orientation of the ~8.8 kb Eco RI insert was determined by digesting appropriate subclones with the restriction endonuclease Xho I. Figure 37 shows the digestion profile of two of these subclones after cleavage with Eco RI and Xho I. The restriction map of λOHNZ1 (Figure 27) indicated that two Xho I sites were located within the ~8.8 kb Eco RI fragment. The Bluescript vector also contained a unique Xho I site in the polylinker (Figure 38) which was used to determine the orientation of the insert in each subclone. Isolate F, a subclone with the insert in a 5'-3' orientation was selected for subsequent analysis.

Isolate F was digested with restriction endonucleases to confirm its identity and to further characterise the insert (Figure 39). Most fragments greater than 2 kb in size appear as broad bands due to excess DNA and accurate sizes could not be determined. This was a likely cause for the variable molecular size totals (Table 18). The Eco RI, Sma I, Sst I and Xho I digestions produced restriction fragments of the predicted sizes (Table 18). Neither Hinc II nor Hind III cleaved within the insert. The single linear fragment visible in these digestions was produced by cleavage at the Hinc II and Hind III restriction sites in the polylinker of the plasmid. Apa I and Pst I cleaved the insert several times producing complex digestion patterns which will require additional experiments to allow interpretation (Table 18). The sizes of the ~9.3, 1.70, 0.47 and 0.22 kb Kpn I fragments agreed with values predicted from the λOHNZ1 map, indicating that the correct ~8.8 kb Eco RI fragment had been cloned. Four of the Bam HI fragments (~0.68, ~2.2, ~1.58 and ~0.3 kb) were mapped according to the location of these fragments within the ~6.4 kb Xba I fragment (Section 3.5.1). These results are illustrated in figure 40.

Figure 37: Restriction analysis of Isolate F, an 8.8 kb Eco RI subclone

Plasmid DNA was prepared by the Wizard Miniprep method (Promega) and digested with the restriction enzymes listed below for one hour at 37°C in the appropriate React® buffer. The digests were subjected to gel electrophoresis through a 1% agarose gel in 1x TAE at 80 V for ~3 hours. DNA fragments were stained with ethidium bromide and visualised under UV illumination.

(1) BRL 1 kb ladder; (2) Isolate F (~1 μg) digested with Eco RI; (3) Isolate F (~1 μg) digested with Xho I; (4) Isolate G (~1.5 μg) digested with Eco RI; (5) Isolate G (~3 μg) digested with Xho I



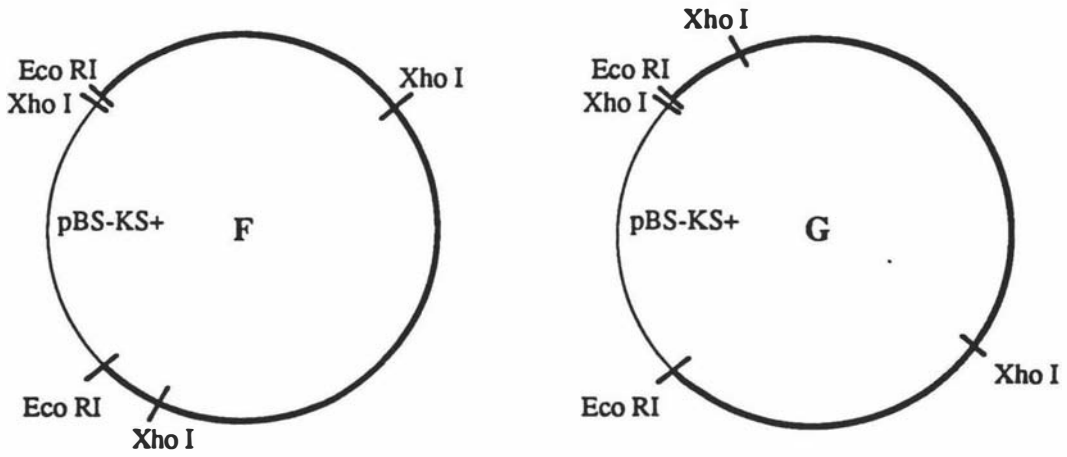


Figure 38: Restriction maps of two possible orientations of the ~8.8 kb Eco RI fragment from λOHNZ1 inserted into the plasmid vector Bluescript® KS+

The orientation of the ~8.8 kb Eco RI fragment was established by digesting the subclones with the restriction enzyme Xho I. Fragments ~4.8, 4.0 and 3.2 kb were characteristic of a 5'-3' orientated construct (Isolate F). Xho I fragments with sizes of ~6.2, 4.8 and 0.8 kb were diagnostic of a 3'-5' orientated insert (Isolate G).

Table 18: Restriction analysis of Isolate F

Digestion fragment sizes were deduced from earlier mapping analysis of the λOHNZ1 clone and isolate A (Sections 3.4 and 3.5.1). The fragment sizes produced by the digestion of isolate F with the restriction endonucleases Apa I, Hinc II and Pst I could not be predicted.

Restriction Endonuclease	Predicted Fragment Sizes(kb)	Observed Fragment Sizes (kb)	Total Molecular Size(kb)
Eco RI	8.8, 2.96	~8.8, ~2.96	11.76
Apa I		~3.2, ~2.8, ~2.2, ~1.15, ~0.68, ~0.57, ~0.3	10.90
Bam HI	2.2, 1.58, 0.68, 0.3	~2.9, ~2.6, ~2.2, ~1.58, ~1.1, ~0.68, ~0.3	11.36
Hinc II		~11.8	11.80
Hind III	11.8	~11.8	11.80
Kpn I	9.30, 1.7, 0.47, 0.22	~9.30, ~1.7, ~0.47, ~0.22	11.69
Pst I		~3.2, ~2.7, ~2.3, ~0.82, ~0.54, ~0.47, ~0.44, ~0.35, ~0.24, ~0.18	11.24
Sma I	4.8, 4.8, 2.2	~4.8, ~4.8,~ 2.2	11.80
Sst I	5.0, 3.95, 2.8	~5.05, ~3.95, ~2.8	11.80
Xba I	5.4, 3.2, 3.2	~5.4, ~3.2, ~2.95	11.55
Xho I	4.8, 3.8, 2.9	~4.8, ~3.8, ~2.9	11.50

Figure 39: Restriction analysis of Isolate F

Plasmid DNA (~2 µg) was digested for one hour at 37°C with the restriction endonucleases listed below in a 30 µl volume using the recommended React® buffers. The digests were analysed by gel electrophoresis on a 0.7% agarose gel in 1x TAE at 90 V for ~3 hours. DNA fragments were stained with ethidium bromide and visualised using a UV transilluminator.

(1) and (13) BRL 1 kb DNA ladder. Isolate F (~2 µg) digested with: (2) Eco RI; (3) ApaI; (4) Bam HI; (5) Hinc II; (6) Hind III; (7) Kpn I; (8) Pst I; (9) Sma I; (10) Sst I; (11) Xba I; (12) Xho I.

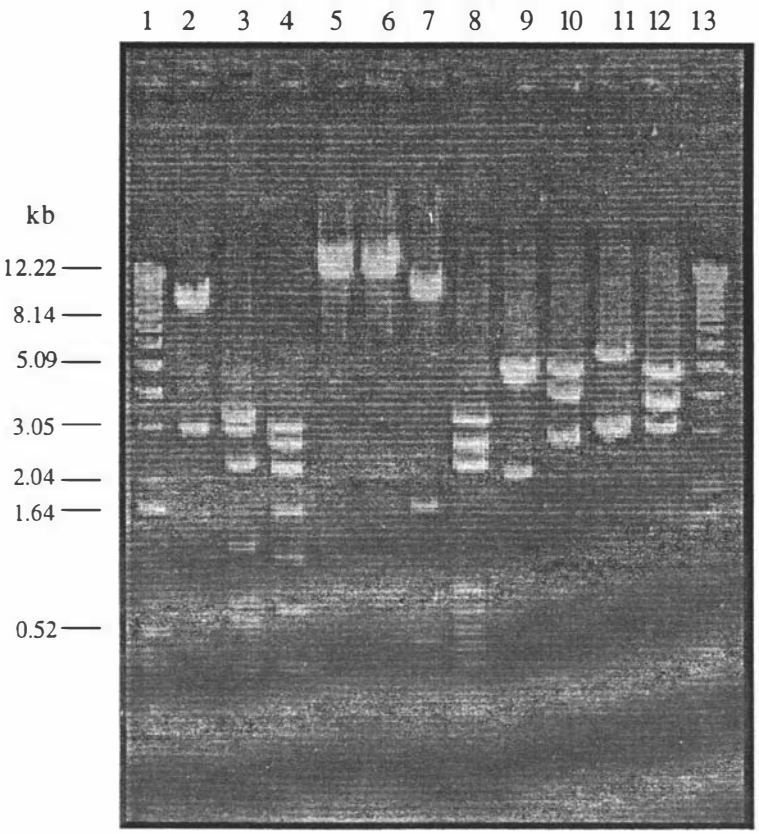
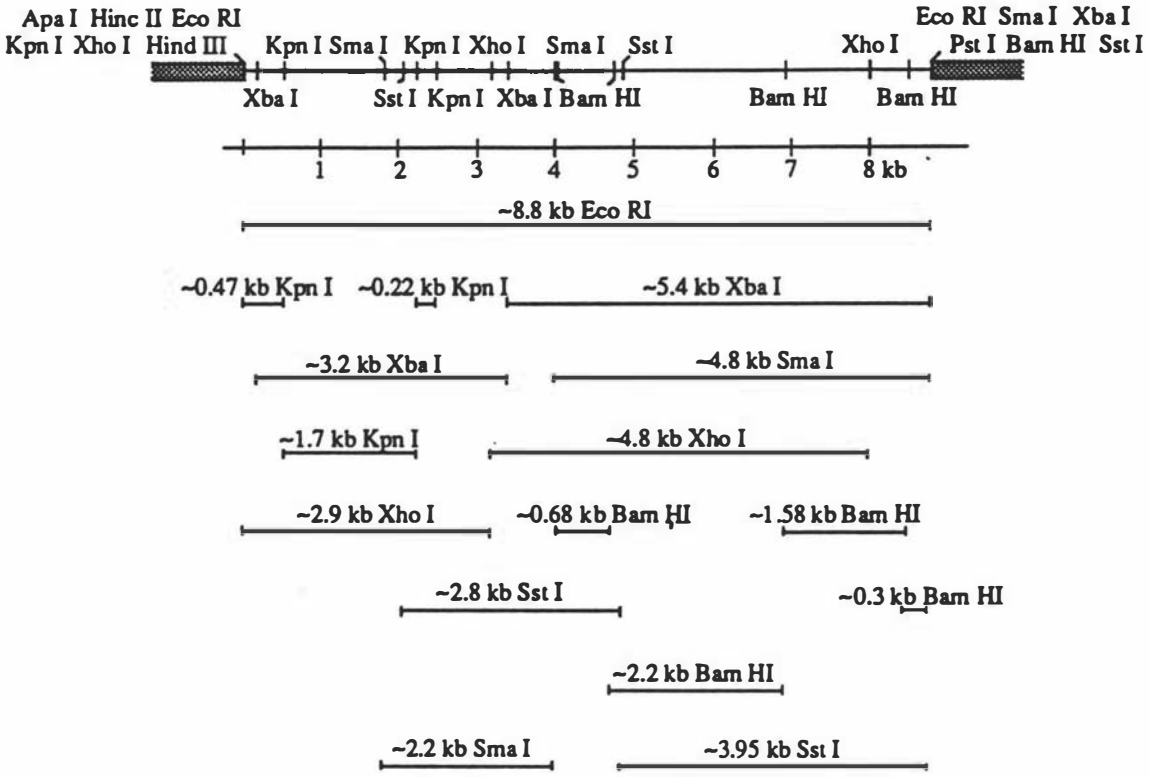


Figure 40: Schematic representation of restriction fragments of Isolate F



Hybridisation of the 8.8 kb Eco RI insert to a probe representing exon I of bovine lactoferrin was investigated by digesting Isolate F with various restriction endonucleases and transferring the resulting fragments to a nylon membrane (Figure 41). Table 19 shows the restriction fragments which were obtained.

Eco RI digestion released a ~8.6 kb insert from the plasmid. Restriction analysis of this insert also indicated a total molecular size of ~8.6 kb (Table 19).

Eco RI cleaved the ~4.8 kb Sma I fragment within the Sma I/Eco RI digestion releasing the vector and a ~1.6 kb fragment. This suggested that the ~4.8 kb doublet observed within the Sma I digestion consisted of a ~4.8 kb fragment and a ~4.6 kb fragment co-migrating. The Sma I/Xba I/Eco RI triple digest was analogous to the Sma I/Xba I digest, as Xba I cleaved within the polylinker and ~0.1 kb from the cloning junction at the other end of the insert. The ~2.0 kb Sma I/Sst I fragment had a slightly lower molecular size than predicted from both the Sma I/Sst I and the Sma I/Sst I/Eco RI digestions. These results are summarised in a basic restriction map of Isolate F shown in figure 42.

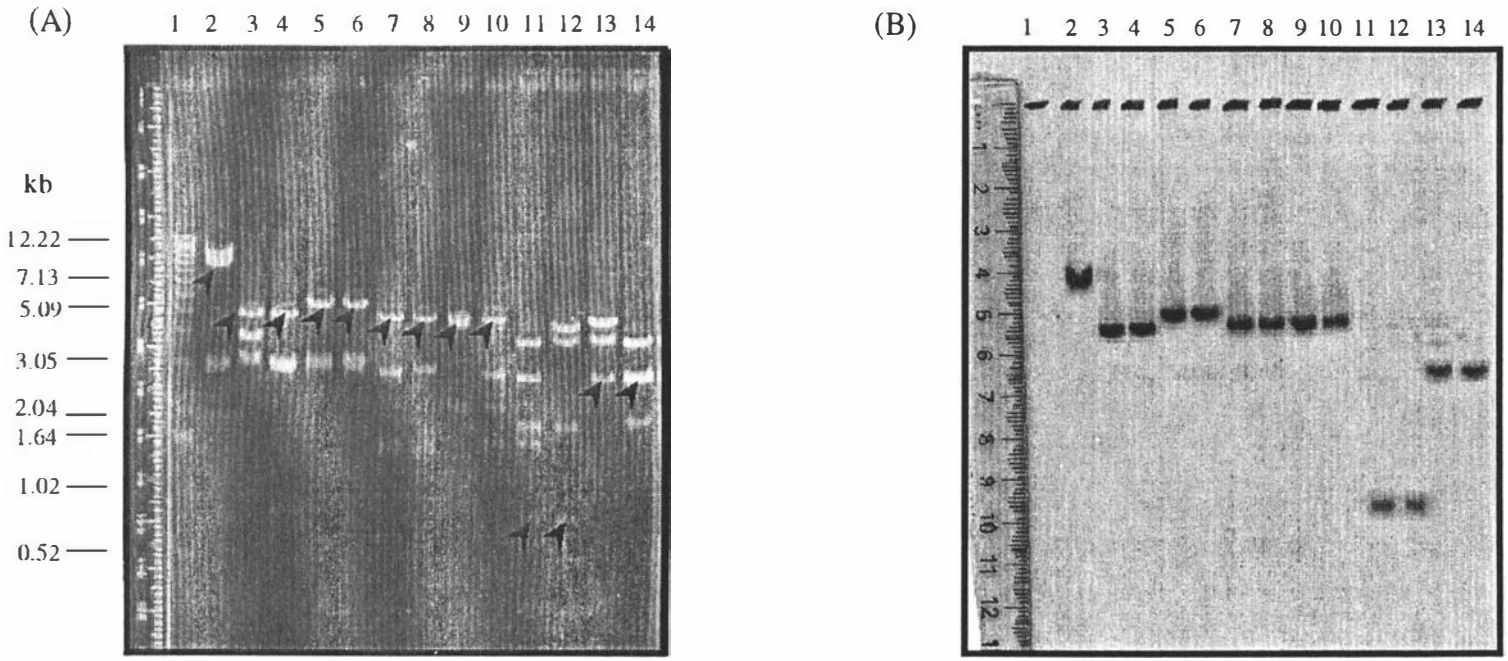


Figure 41: Restriction and Southern analysis of plasmid DNA of Isolate F

(A). Plasmid DNA was prepared by the Wizard Miniprep DNA method (Promega) and was digested with the restriction endonucleases listed below for one hour at 37°C in the appropriate buffer. The digests were analysed by gel electrophoresis on a 0.7% agarose gel in 1x TAE. The DNA bands were stained with ethidium bromide and visualised under UV illumination. Arrows indicate the digestion fragments which hybridised to the probe.

(B). The digested DNA (A) was transferred to nylon membrane by Southern blotting. The membrane was hybridised at 68°C with a radiolabelled probe derived from exon I sequences and washed under conditions of high stringency (68°C, 1x SSC). The washed filter was exposed to X-ray film for seven minutes at room temperature.

(1) BRL 1 kb DNA ladder. Isolate F DNA (1.5 µg) digested with: (2) Eco RI; (3) Xho I; (4) Eco RI/Xho I; (5) Xba I; (6) Xba I/Eco RI; (7) Sma I/Xba I/Eco RI; (8) Sma I/Xba I; (9) Sma I; (10) Eco RI/Sma I; (11) Sma I/Sst I/Eco RI; (12) Sst I/Sma I; (13) Sst I; (14) Eco RI/Sst I

Table 19: Restriction Analysis of the 8.6 kb Eco RI subclone

Digestion fragment sizes were determined from the mapping analysis of λOHNZ1 (Section 3.4)

Restriction Endonuclease	Predicted Fragment Sizes (kb)	Observed Fragment Sizes (kb)	Total Molecular Size (kb)
Eco RI	8.6, 2.96	~8.6, ~2.95	11.55
Xho I	4.8, 3.8, 2.95	~4.8, ~3.8, ~2.9	11.50
Eco RI/Xho I	4.8, 2.96, 2.95, 0.8	~4.8, ~2.95, ~2.95, ~0.83	11.53
Xba I	5.4, 3.2, 2.95	~5.4, ~3.1, ~2.95	11.45
Xba I/Eco RI	5.4, 3.2, 2.96	~5.4, ~3.2, ~2.95	11.55
Sma I/Xba I/Eco RI	4.8, 2.96, 1.6, 1.5, 0.6	~4.8, ~2.9, ~1.6, ~1.43, ~0.62	11.35
Sma I/Xba I	4.8, 2.96, 1.6, 1.5, 0.6	~4.8, ~2.95, ~1.6, ~1.43, ~0.62	11.40
Sma I	4.8, 4.6, 2.2	~4.8, ~4.8, ~2.2	11.80
Eco RI/Sma I	4.8, 2.96, 2.2, 1.6	~4.8, ~2.95, ~2.2, ~1.6	11.55
Sma I/Sst I/Eco RI	3.9, 2.96, 2.0, 1.6, 0.8, 0.2	~3.9, ~2.9, ~1.9, ~1.6, ~0.8, ~0.22	11.32
Sst I/Sma I	4.6, 3.9, 2.0, 0.8, 0.2	~4.65, ~3.9, ~1.85, ~0.8, ~0.22	11.42
Sst I	4.8, 3.9, 2.9	~4.8, ~3.9, ~2.9	11.60
Eco RI/Sst I	3.9, 2.96, 2.9, 1.8	~3.9, ~2.95, ~2.9, ~1.8	11.55

Figure 41B shows the autoradiograph produced by the hybridisation of a probe representing exon I sequences of bovine lactoferrin to the digested plasmid DNA. A single specific hybridisation signal was detected in each restriction digestion. These results are shown in table 20.

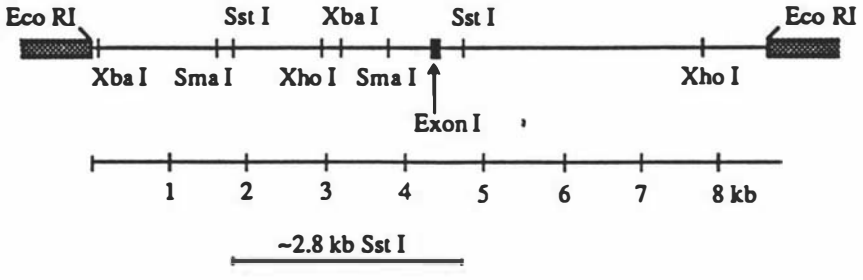
Table 20: Hybridisation analysis of ~8.6 kb Eco RI subclone

Hybridisation fragment sizes were predicted from the mapping analysis of λOHNZ1 (Section 3.4)

Restriction Endonuclease	Predicted Hybridisation Fragment Size (kb)	Observed Hybridisation Fragment Size (kb)
Eco RI	8.6	~8.6
Xho I	4.8	~4.8
Eco RI/Xho I	4.8	~4.8
Xba I	5.4	~5.4
Xba I/Eco RI	5.4	~5.4
Sma I/Xba I/Eco RI	4.8	~4.8
Sma I/Xba I	4.8	~4.8
Sma I	4.8	~4.8
Eco RI/Sma I	4.8	~4.8
Sma I/Sst I/Eco RI	0.8	~0.8
Sst I/Sma I	0.8	~0.8
Sst I	2.9	~2.9
Eco RI/Sst I	2.9	~2.9

The data obtained from restriction digestions and Southern hybridisation analysis of isolate F was consistent with the restriction map deduced earlier for this region of the λOHNZ1 clone. These results indicate that the insert within isolate F was analogous to the ~8.8 kb fragment produced by the cleavage of λOHNZ1 with Eco RI. Figure 42 shows that ~4.0 kb of DNA lies 5' to exon I within isolate F. A ~2.8 kb Sst I fragment from this region of DNA (Figure 42) was selected for dideoxy sequence analysis as this fragment should contain the proximal promoter of the bovine lactoferrin gene.

Figure 42: Basic restriction map of Isolate F



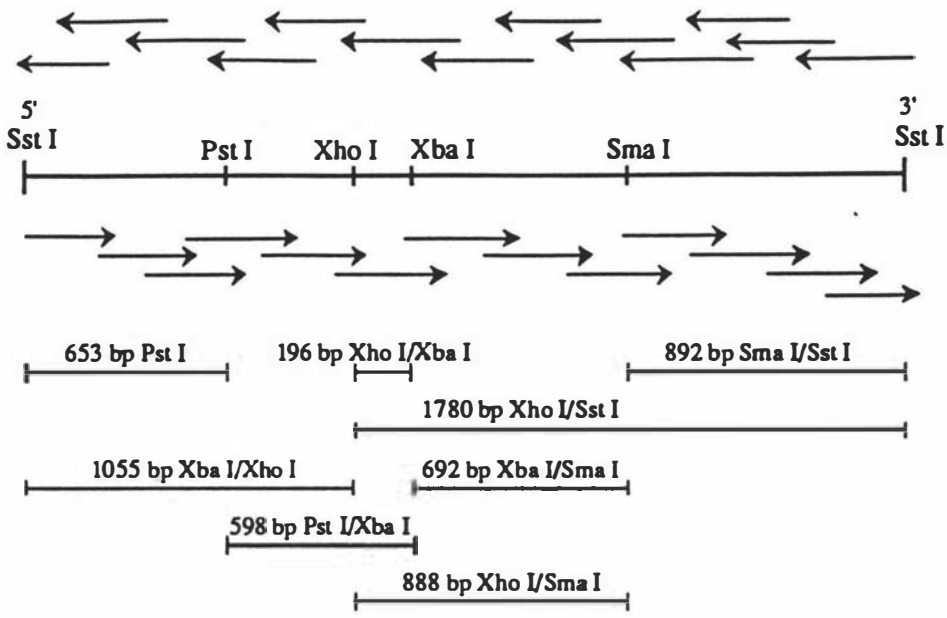
3.5.4 Characterisation of the 2.8 kb Sst I fragment

A ~2.8 kb Sst I fragment had been shown previously to hybridise to a radiolabelled probe derived from sequences corresponding to exon I (Sections 3.4 and 3.5.3). This fragment was subcloned into the plasmid vector Bluescript™ and sequenced using the dideoxy chain termination method as described in section 2.2.18.

Overlapping restriction fragments within the 2.8 kb Sst I fragment were subcloned into Bluescript® to facilitate the sequence analysis. Both strands of each subclone were sequenced at least twice. The oligonucleotide primers used were designed from sequence data obtained from the 2.8 kb fragment and were positioned approximately 250 bp apart. The sequencing strategy is illustrated in figure 43 and the sequence which was obtained is shown in Appendix 4.

Seyfert *et al.* (1994) recently reported the isolation of 1007 bp of bovine lactoferrin promoter sequence. Alignment of this sequence with the corresponding region of sequence of the 2.8 kb Sst I fragment showed 99.283% sequence homology, as determined by the Genetics Computer Group alignment programme 'Bestfit' (Appendix 5). Allelic differences are likely to account, in part, for the minor variations observed between these two sequences.

Figure 43: Sequencing strategy for the 2.8 kb Sst I fragment



3.5.4.1 Comparison of the Promoter Sequences from Mouse, Human and Bovine Lactoferrin

Figure 44 shows the nucleotide alignment of ~800 bp of promoter and exon I sequence from mouse, human and bovine lactoferrin. The bovine lactoferrin sequence (Figure 44) exhibited a higher degree of sequence homology to the human lactoferrin promoter region (~63%) than the corresponding mouse lactoferrin sequences (~54%). Both the human and mouse lactoferrin promoters contain COUP/ERE (Wang *et al.*, 1987; Klein-Hitpass *et al.*, 1988), Pu boxes (Pettersson and Schaffner, 1987), CAAT (Thalmeier *et al.*, 1989), Sp1 (Jackson *et al.*, 1990) and TATA (Wefald *et al.*, 1990) transcription elements (Teng *et al.*, 1992). Comparison of the human, bovine and mouse lactoferrin promoters (Figure 44) indicated that the bovine lactoferrin sequence contained a TATA sequence element (ATAAA) at nucleotide -29, a putative Sp1 element (GGGCGGGG) at nucleotide -64 and a putative Pu box (ATCCTC) at nucleotide -221 relative to the start site of transcription (Goodman & Schanbacher, 1991). No homology was observed between the bLf promoter sequence and the region within the mouse (nt -349 to -329) and human (nt -362 to -342) lactoferrin promoters which contain an estrogen responsive/COUP-TF element (Teng *et al.*, 1992). Seyfert *et al.* (1994) proposed that the lack of this sequence element and a GATA-1 binding element might explain the comparatively low level of expression *in vivo* of the bovine lactoferrin gene compared to the human lactoferrin gene.

Putative transcription factor binding sites can be identified within promoter sequences by using computer sequence homology programmes. The results obtained from such a computer search provide a preliminary indication of putative binding interactions between the DNA sequence and known protein transcription factors. Homologies with the consensus sequences for the transcription factors GATA-1, Sp1, Oct-1, nuclear factor-kappa B (NF-κB), cyclic AMP response element (CRE), glucocorticoid response element (GRE), progesterone receptor, nuclear factor-interleukin-6 (NF-IL6), GC factor (GCF), activator protein-1 (AP-1) and TATA were identified within the sequence obtained from the ~ 2.85 kb Sst I fragment (Faisst & Meyer, 1992) (Figure 45). Before the significance of these putative transcription factor binding sites can be ascertained, specific binding of the transcription factor to the promoter sequence will need to be demonstrated. This could be achieved by analyses such as DNase I footprinting and electrophoretic gel mobility shift assays. Before these investigations could be undertaken, it was critical to demonstrate that the isolated fragment of DNA represented a functional promoter. This was carried out by investigating the ability of the putative bovine lactoferrin promoter to direct the expression of a heterologous reporter gene *in vitro*.

Figure 44: Nucleotide alignment of promoter and exon I regions of mouse (M), bovine (B), and human (H) lactoferrin

Mouse lactoferrin sequence (M74778) was determined by Liu and Teng (1991). The human lactoferrin sequence (S52659) was deduced from human placental tissue by Teng *et al.* (1992). The sequences were aligned using the University of Wisconsin Genetics Computer Group Vax programme Bestfit.

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M   -717 TAGACACAGACAGACACACAGAGGAAGAGGAAAAAAAGATATGGAGGAAGGAATGAAAGAAAAGGAGCAAAAGAAATCAAATGTCAGGGTCTCTTCCTCG -619
B   -618 ..........AGGACAAAATAGGACATTTATCAAAATGAGGTTCCTGTCTCCCACCTCATATTGCCACAAAACAACACAAGGGGTAGGATATCCTTTTCA -680
H   -764 AATACTATCAACACCAAAAAAGAAGAAGGAGGAGATGGAGAAAAAAAAAGACAAAAAAAAAAAAAAAGTGGTAGGGCATCTTAGCCATAGGGCATCTTTCTCA -666

M   -618 CTAGCAAATGAAGGGACACAGGTCAACCCTGGG..........TGGCATTCCTTTCTGAGGTCCTAGGTTATTTTCGGGGGCTGTATGG.CGGGTTTCAA -529
B   -679 TTGGCAAATGAGGGACCAGGAGACAGCCTTTGG..........GCACTTAGGCCTCTG......GTTCTGTTTTCTGGGAGCTGTATTG.CGGTCT.... -600
H   -665 TTGGCAAAT.AAGAACATGGAACCAGCCTTGGGTGGTGGCCATTCCCCTCTGAGGTCCC......TGTCTGTTTTCTGGGAGCTGTATTGTGGGTCT.... -575

M   -528 GGCAGTGTGG..........ACCCCACAGGAACCCTGTGTGCAAGTCTAGGCTGACTCCGCTCTCCCTGCGGCTGTCACCGGGCTGCTGTTGTTGGCCAG -438
B   -599 ...CAGGAGG..........ACCCCAGGGGCAGTCTGGGTCAGACTCTGGGCAGCCTCTGCCAGCTGGACCAGGCTGCCGTGGACCCCG....GGCCAGG -516
H   -574 ...CAGCAGGGCAGGGAGATACCCCATGGGCAGCTTGCCTGAGACTCTGGGCAGCCTCTCTTTTCTCTGTCAGCTGTCCCTAGGCTGCT....GCTGGGG -481

M   -437 GCC.TGAGCAGCTGCCTCTTCTTTAGAATCCACCACTCTTTGTCTAGCCAAGGAGGAAGG...GGATTTGCTTGCTCCATGCAGCTTAAG......TGT -348
B   -515 CAG..CGGGCCCTCTTTCAAAACTCCAGGCTGGCTCTGCGTGCAGATGCAAGGGTCTCCGTCTGTCTTAACTGGTTCCCAAGCACTTTAGATACCTTCT -418
H   -480 GTGGTCGGGTCATCTTTTCAACTCTCAGCTCACTGCTGAGCCAAGGTGAAAGCAAACCCACCTGCCCTAACTGGCTCCTAGGCACCTTCAAGGTCATCT -381

M   -347 .............CACAGGTCAAGGTAACCCACAAATATAGACCCCCTACCCCATG........TCCCACCTCTAGAAAGTACTGGAAACAGAGAAAGG -269
B   -417 .............CTATAGTCAAGCTGATCCGCAAAGATTCACCCTAGGACCCCTGCTCTGGATCCCGCTCTCTAGGAGGCACTGAGACCGGAGCGGGG -331
H   -380 GCTGAAGAAGATAGCAGTCTCACAGGTCAAGGCGATCTTCAAGTAAAGACCCTCTGCTCTGTGTCCTGCCCTCTAGAAGGCACTGAGACCAGAGCTGGG -281
```

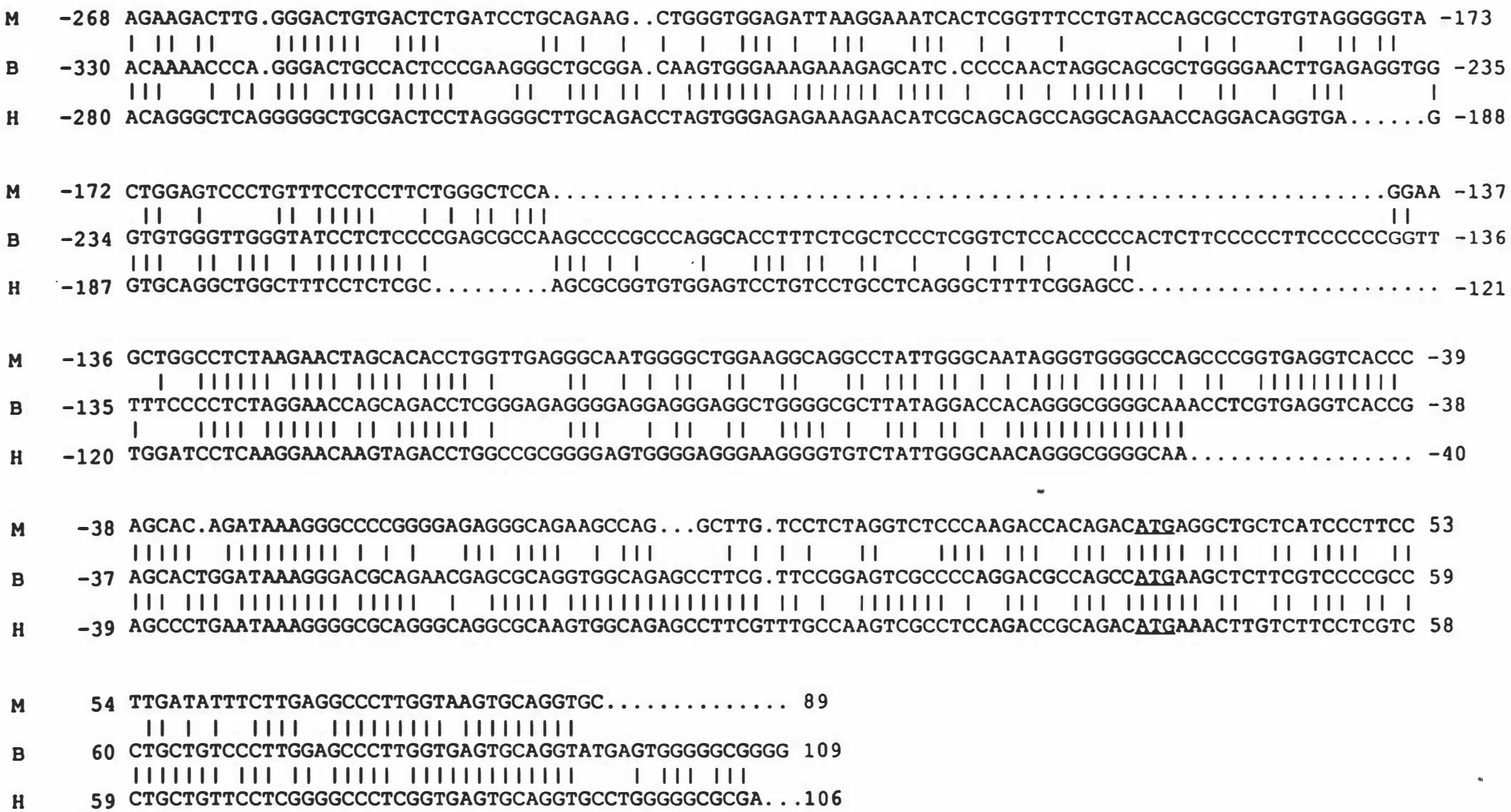

M -268 AGAAGACTTG.GGGACTGTGACTCTGATCCTGCAGAAG..CTGGGTGGAGATTAAGGAAATCACTCGGTTTCCTGTACCAGCGCCTGTGTAGGGGGTA -173
B -330 ACAAAACCCA.GGGACTGCCACTCCCGAAGGGCTGCGGA.CAAGTGGGAAAGAAAGAGCATC.CCCCAACTAGGCAGCGCTGGGGAACTTGAGAGGTGG -235
H -280 ACAGGGCTCAGGGGGCTGCGACTCCTAGGGGCTTGCAGACCTAGTGGGAGAGAAAGAACATCGCAGCAGCCAGGCAGAACCAGGACAGGTGA.....G -188
M -172 CTGGAGTCCCTGTTTCCTCCTTCTGGGCTCCA.....GGAA -137
B -234 GTGTGGGTTGGGTATCCTCTCCCCGAGCGCCAAGCCCCGCCCAGGCACCTTTCTCGCTCCCTCGGTCTCCACCCCCACTCTTCCCCCTTCCCCCCGGTT -136
H -187 GTGCAGGCTGGCTTTCCTCTCGC.....AGCGCGGTGTGGAGTCCTGTCCTGCCTCAGGGCTTTTCGGAGCC..... -121
M -136 GCTGGCCTCTAAGAACTAGCACACCTGGTTGAGGGCAATGGGGCTGGAAGGCAGGCCTATTGGGCAATAGGGTGGGGCCAGCCCGGTGAGGTCACCC -39
B -135 TTTCCCCTCTAGGAACCAGCAGACCTCGGGAGAGGGGAGGAGGGAGGCTGGGGCGCTTATAGGACCACAGGGCGGGGCAAACCTCGTGAGGTCACCG -38
H -120 TGGATCCTCAAGGAACAAGTAGACCTGGCCGCGGGGAGTGGGGAGGGAAGGGGTGTCTATTGGGCAACAGGGCGGGGCAA..... -40
M -38 AGCAC.AGATAAAGGGCCCCGGGGAGAGGGCAGAAGCCAG...GCTTG.TCCTCTAGGTCTCCCAAGACCACAGACATGAGGCTGCTCATCCCTTCC 53
B -37 AGCACTGGATAAAGGGACGCAGAACGAGCGCAGGTGGCAGAGCCTTCG.TTCCGGAGTCGCCCCAGGACGCCAGCCATGAAGCTCTTCGTCCCCGCC 59
H -39 AGCCCTGAATAAAGGGGCGCAGGGCAGGCGCAAGTGGCAGAGCCTTCGTTTGCCAAGTCGCCTCCAGACCGCAGACATGAAACTTGTCTTCCTCGTC 58
M 54 TTGATATTTCTTGAGGCCCTTGGTAAGTGCAGGTGC..... 89
B 60 CTGCTGTCCCTTGGAGCCCTTGGTGAGTGCAGGTATGAGTGGGGGCGGGG 109
H 59 CTGCTGTTCCTCGGGGCCCTCGGTGAGTGCAGGTGCCTGGGGGCGCGA...106

Figure 45: Putative transcription factor binding sites within the bovine lactoferrin gene

The consensus binding sequences for the given transcription factors (Faisst & Meyer, 1992) are shown above the region of the lactoferrin promoter exhibiting homology to these elements. The position of these putative binding sites was deduced using the GCG VAX programme 'Findpatterns'. The numbering refers to the position within the bovine lactoferrin sequence shown in Appendix 4. Putative binding interactions to both strands are shown. Mismatchs have been allowed (eg. mis=2) for some transcription factors.

```
                    AGATAR                  (GATA-1 consensus)
          154: TTGGT AGATAG GTAAC

Reverse             TTASTCA
          801: GAGAA TTAGTCA ATTTT

                    TGATAR                  (GATA-1 consensus)
          415: AATGT TGATAA CAGAT

Reverse             YTATCA
        1,717: GACAT TTATCA AAATG

                    SCGSSSC                 (GC-Factor consensus)
        1,942: GGACC CCGGGCC AGGCA
        1,954: AGGCA GCGGGCC CTCTT
        2,401: ACAGG GCGGGGC AAACC

Reverse             GSSSCGS
        1,134: CAACA GGGCCGC CTCCT          R=A or G
        2,264: GCCAA GCCCCGC CCAGG          Y=C or T
        2,379: AGGCT GGGGCGC TTATA          S=G or C
        2,564: GAGTG GGGGCGG GGGCA          N=A or G or C or T
        2,607: CCCAC GCCCCGC TGTGG          K=G or T
        2,733: AGGAG GCGCCGG GAGCG          M=A or C

                  TKNNGNAAK                 (NF-IL6 consensus)
          828: ATTAT TTTTGTAAT GGCTT
          853: GTTAT TGATGAAAG CAACT
        1,490: ACACA TGCTGCAAT GGAAG

Reverse           MTTNCNNMA
          546: GTCTG CTTACTCCA ATCCT
        1,232: GACAG ATTTCAGAA TAACA
        1,750: CTCAT ATTGCCACA AAACA
        1,963: GCCCT CTTTCAAAA CTCCA

                  KRGGCKRRK                 (Sp1 consensus)
          503: GGCCT GAGGCTGGG ACATT
        2,373: GGAGG GAGGCTGGG GCGCT
        2,564: GAGTG GGGGCGGGG GCATG

Reverse           MYYMGCCYM
          581: CTCAG CTCAGCCTC TCAGT
        1,665: TGAAA ACCAGCCTC CTGAA
        2,265: CCAAG CCCCGCCCA GGCAC

                  ATGCAAAT                  (Oct-1 consensus)
          223: TGATG atgcaaac CGGTC mis=1
```

```
Reverse                ATTTGCAT              (Oct-1 consensus)
          686: TTCTA atttgcac TTGGA mis=1
        1,338: TCAGT acttgcat GGTGG mis=1

                       TATAAA                (TATA consensus)
          735: TCTTT tataat CACGG mis=1
          773: TTAGA tacaaa GATGC mis=1
        1,420: TTTTT tttaaa CGTTT mis=1
        1,718: ACATT tatcaa AATGA mis=1
        2,434: CACTG gataaa GGGAC mis=1

Reverse                TTTATA
          270: CTCTA tttatg CTGCC mis=1
          414: GAATG ttgata ACAGA mis=1
          680: TCCTT tttcta ATTTG mis=1
          733: ATTCT TTTATA ATCAC
          767: GCTAA tttaga TACAA mis=1
          829: TTATT tttgta ATGGC mis=1
        1,418: CCTTT ttttta AACGT mis=1
        1,420: TTTTT tttaaa CGTTT mis=1
        1,652: CTAGC tttaga ACTGA mis=1
        1,716: GGACA tttatc AAAAT mis=1
        2,035: AGCAC tttaga TACCT mis=1
        2,048: CCTTC tctata GTCAA mis=1
        2,385: GGGCG cttata GGACC mis=1

                       GGGAMTNYCC            (NF-kB consensus)
          509: AGGCT gggacattcc TTGGA mis=1
        1,269: ACAGA gggaatttct CTCAC mis=1
        1,615: TTCAA gggagtgtcc TTCAA mis=1
        2,145: ACCCA gggactgcca CTCCC mis=1
        2,571: GGCGG gggcatgccc CTCCA mis=1

Reverse                GGRNAKTCCC
          445: CTTCT gggcagcccc ACCTC mis=1
          510: GGCTG ggacattcct TGGAG mis=1
          567: CTCCA gggcaatccc TCAGC mis=1
        1,509: CGCCA gggaagtcct CCCCC mis=1
        2,571: GGCGG gggcatgccc CTCCA mis=1

                       TGTTCACT              (Progesteronc Receptor consensus)
        1,299: TGACT tcttcact TAGTA mis=1

Reverse                AGTGAACA
        1,557: CAATC agtgaacg ATAAG mis=1

                       GTWCANNNTGTYCT        (GRE consensus)
          431: CCATG gaacattgtcttct GGGCA mis=2
          606: CCCAG gttctctatgttcc TGCCA mis=2

                       TGACGTCA              (CRE consensus)
          320: CGGTG tgccgtca GCTCC mis=1
        2,416: CCTCG tgaggtca CCGAG mis=1

                       TGAGTCA               (AP-1 consensus)
          396: TTGGA tgagtga ATGAA mis=1
          801: GAGAA ttagtca ATTTT mis=1
        1,891: CAGTC tgggtca GACTC mis=1
```

3.6 Sequence Analysis of Exon II of Bovine Lactoferrin

Hybridisation analyses of clone I and λOHNZ1 indicated that exon II sequences of bovine lactoferrin were contained within non-identical restriction endonuclease fragments (Sections 3.1 and 3.4). The fragments of interest are described in Table 21. To resolve these discrepancies, two fragments containing exon II (~810 bp Xho I/Eco RI, ~1.58 kb Bam HI) were isolated from the λOHNZ1 clone and subjected to dideoxy sequence analysis as described in section 2.2.18.

Table 21: Digestion fragments containing exon II sequences of bovine lactoferrin

	Restriction Fragment	
Clone	Eco RI	Bam HI
Clone I (Clonetech)	2.025 kb	0.531 kb
λOHNZ1 (Stratagene)	~8.8 kb	~1.58 kb

Sequence analysis of fragments isolated from clone I and λOHNZ1, showed that both of these clones contained sequences for exon II of bovine lactoferrin (Figure 46). Both of these contained the C→T base substitution which had been identified previously by the comparison of the bovine lactoferrin cDNA sequence with sequence obtained from clone I (Appendix 2; Section 3.1). Comparison of the sequence data (Appendix 1) showed that the sequence within λOHNZ1 and clone I diverged in the region 5' to exon II (Figure 46). Sequences 3' to exon II were 100% homologous which indicated that this DNA was likely to be part of intron II of bovine lactoferrin.

The variation in restriction fragment sizes observed between clone I and λOHNZ1 (Table 21) was likely to be a direct consequence of the DNA sequence heterogeneity. Clearly, one or both of these clones contained erroneous DNA sequences which may have been introduced during the construction of the genomic library.

Figure 46: Sequence homology of exon II fragments of bovine lactoferrin

The nucleotide sequence from the Eco RI fragment isolated from clone I (nt 1011-2025) compared to the sequence from fragments derived from λOHNZ1 (nt 1-879). Exon II sequences are shown in uppercase letters.

```
   1 ..............................agcttccgcaccagaaaga 19
                                    || |     |      |
1101 gggtcagagccagagccccacacgggcagaccgcgtttcatcacactcgg 1150

  20 cagacgcaatactttctctctgcctacctcctaggttccaggggatcaaa 69
     ||| |    |  ||| |  |  || |     |  |        |     |
1151 caggcatgctggtttttaccaccccaaggtttgtgacaaccctggatcca 1200

  70 gcaggtggaagaactcccttagctgatgcaagggagcattgctggctttt 119
     ||| ||  |      ||| |   |    ||        |  || | | |
1201 gcaagtttatttggccccgtttttccaacagcactcgctcactcggtgtc 1250

 120 tgtcgg..............gggtaggaagtatgtcagtcagaggagg 153
     | | ||                    |  | |       ||      |
1251 tctgggtcacgttttgataattttcacgttatttccagcttcttcattgc 1300

 154 gaaacgattccagaggctctgatcatgtcctcaaacagccctctggccag 203
      | |    |   |  | | ||||||||||||||||||||||||||||||
1301 tatatttgtggtggtgatgtgatcatgtcctcaaacagccctctggccag 1350

 204 catggagtggtcattaggctagcgaaacacctccatcagaggagagtggc 253
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1351 catggagtggtcattaggctagcgaaacacctccatcagaggagagtggc 1400

 254 ctgactcagccacagaatggagagggaaggggagggcccctgcttgagag 303
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1401 ctgactcagccacagaatggagagggaaggggagggcccctgcttgagag 1450

 304 ggcaaggccgtctcttccacggacagtgatagagccctcactctttggcc 353
     ||||| ||||||||||||||||||||||||||||||||||||||||||||
1451 ggcaa.gccgtctcttccacggacagtgatagagccctcactctttggcc 1499

 354 tctttctcccagGACTGTGTCTGGCTGCCCCGAGGAAAAACGTTCGATGG 403
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1500 tctttctcccagGACTGTGTCTGGCTGCCCCGAGGAAAAACGTTCGATGG 1549

 404 TGTACCATCTCCCAACCTGAGTGGTTCAAATGCCGCCGATGGCAGTGGAG 453
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1550 TGTACCATCTCCCAACCTGAGTGGTTCAAATGCCGCCGATGGCAGTGGAG 1599

 454 GATGAAGAAGCTGGGTGCTCCCTCTATCACCTGTGTGAGGAGGGCCTTTG 503
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1600 GATGAAGAAGCTGGGTGCTCCCTCTATCACCTGTGTGAGGAGGGCCTTTG 1649

 504 CCTTGGAATGTATCCGGGCCATCGCGgtgagtccaggccgtaggctgggt 553
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1650 CCTTGGAATGTATCCGGGCCATCGCGgtgagtccaggccgtaggctgggt 1699

 554 gggaccagactgaaagggaagggatccaagtgccatggaaaatgtgctcg 603
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1700 gggaccagactgaaagggaagggatccaagtgccatggaaaatgtgctcg 1749

 604 ccctgccccctctgctcttcttcccgagcgcctgtaagttgggagctgt 653
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1750 ccctgccccctctgctcttcttcccgagcgcctgtaagttgggagctgt 1799
```

```
 654 cctctctcctaggacacagcgttattttggaagcagacattgccattcta 703
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1800 cctctctcctaggacacagcgttattttggaagcagacattgccattcta 1849

 704 acatttgaaaaccatctgtagggaaactggcatcaccacacaaattgaga 753
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1850 acatttgaaaaccatctgtagggaaactggcatcaccacacaaattgaga 1899

 754 ataatgacggtgcaacgatggtggctcagatggtaaagaatccacctgca 803
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1900 ataatgacggtgcaacgatggtggctcagatggtaaagaatccacctgca 1949

 804 atgcaggacacctgggttcgatccctgggttgggaagatcccctggagaa 853
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1950 atgcaggacacctgggttcgatccctgggttgggaagatcccctggagaa 1999

 854 ggaaatggcaatgcactccagaattc 879
     |||||||||||||||||||||||||
2000 ggaaatggcaatgcactccagaattc 2025
```

Southern analysis of genomic DNA was carried out to determine the authentic sizes of Eco RI and Bam HI fragments in the bovine genome which hybridise to exon II of lactoferrin.

3.7 Analysis of Bovine Genomic DNA

Bovine genomic DNA was isolated from white blood cells and was digested to completion by restriction endonucleases as described in sections 2.2.12 and 2.2.13. The samples were separated by gel electrophoresis through a 0.7% agarose gel (Figure 47) and transferred to nylon membrane according to Southern (1975).

The digested genomic DNA was analysed by the hybridisation of probes derived from exon I and exon II of the bovine lactoferrin gene. These regions represent the 5' most regions of the bovine lactoferrin cDNA sequence and are therefore closest to the promoter region. Oligonucleotide primers complementary to the 5' and 3' ends of the individual exon regions were used for the amplification of exon I and exon II of bovine lactoferrin by *Taq* DNA polymerase (Section 2.2.17). The PCR products were radiolabelled and hybridised to the membranes containing the genomic digests (Section 2.2.9). The membranes were washed and exposed to X-ray film at -70°C using intensifying screens.

Analysis of exon I of lactoferrin from bovine genomic DNA

The autoradiograph produced by the hybridisation of exon I sequences from bovine lactoferrin to genomic DNA is shown in figure 47. Discrete hybridisation signals were visible in all lanes containing genomic DNA. The sizes of Bam HI,

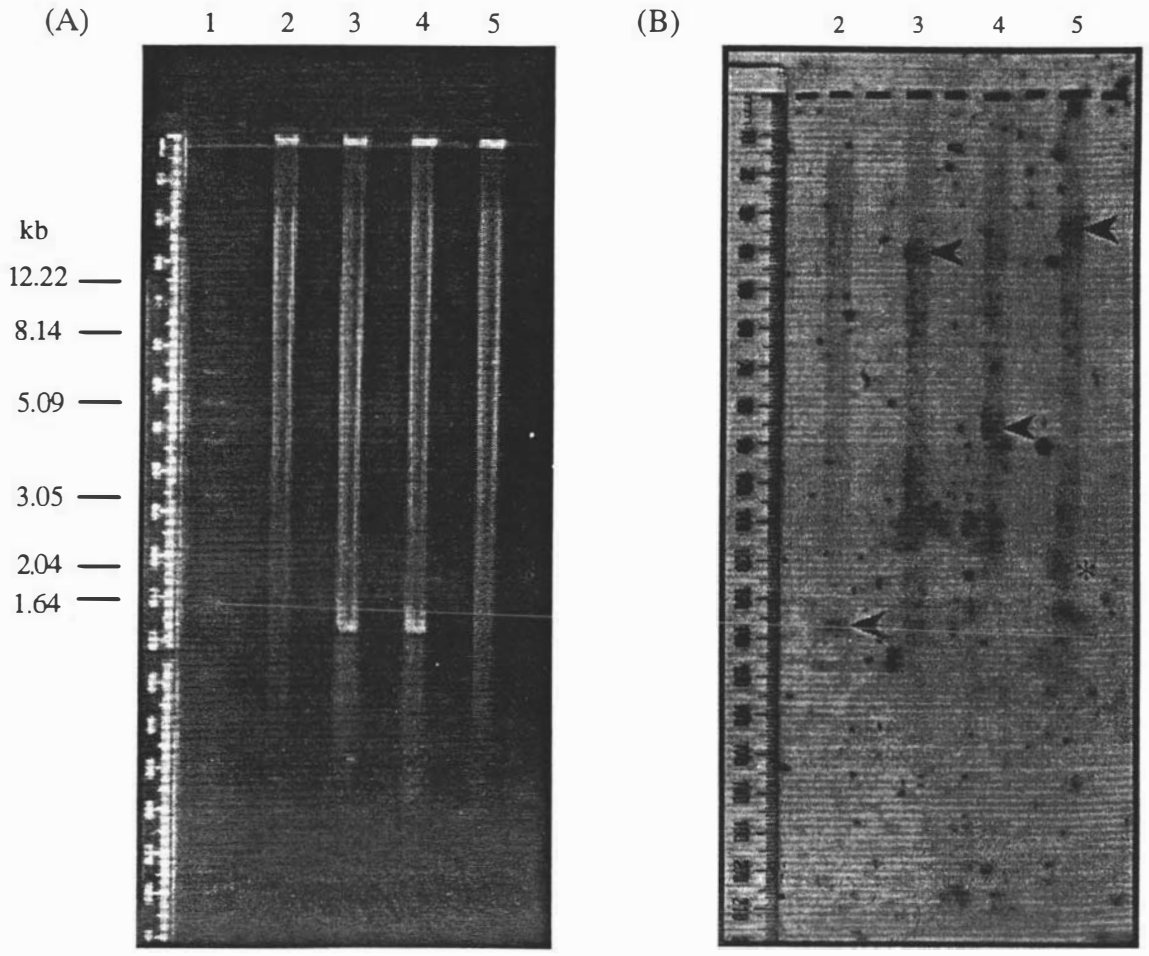


Figure 47: Agarose gel electrophoresis and Southern blot hybridisation analysis of bovine genomic DNA using a radiolabelled probe derived from exon I sequences of bovine lactoferrin.

(A). Gel photograph of genomic DNA which had been digested to completion.
(B). Autoradiograph of Southern blot hybridsed with a radiolabelled probe derived from exon I. The 1 kb DNA ladder (Lane 1) was removed prior to Southern transfer as this was known to hybridise to radiolabelled probes derived from bovine lactoferrin sequences (Figure 6). Specific hybridisation signals are indicated by an arrowhead. A non-specific hybridisation signal (*) was visible in lane 5.

(1). BRL 1 kb DNA ladder. (2). Genomic DNA (~20 µg) digested with Bam HI,
(3). Genomic DNA (~20 µg) digested with Eco RI, (4). Genomic DNA (~20 µg) digested with Sst I,
(5). Genomic DNA (~20 µg) digested with Xba I

Eco RI and Sst I fragments which hybridised to exon I agreed with values predicted from earlier mapping analysis of λOHNZ1 (Table 22). The ~11.5 kb Xba I fragment which hybridised to exon I was larger than the expected ~6.4 kb Xba I fragment present in λOHNZ1. The most likely explanation of this discrepancy is as follows. One of the restriction sites producing the ~6.4 kb Xba I fragment is located on the left arm of the λFIX II vector. Therefore, it is likely that Sau 3A, which was used to generate the fragments used to construct the Stratagene λFIX II library, cleaved between two Xba I restriction sites in the bovine genomic DNA.

Table 22: Analysis of genomic DNA containing sequences representing exon I of bovine lactoferrin

Sizes of fragments which hybridised were predicted from mapping analysis of λOHNZ1 and fragments isolated from this clone (Sections 3.4, 3.5.1 and 3.5.3).

Restriction endonuclease	Predicted hybridisation fragment size (λOHNZ1)	Observed hybridisaton fragment sizes
Bam HI	0.67	~0.65 kb
Eco RI	8.8 kb	~8.8 kb
Sst I	2.85 kb	~2.9 kb
Xba I	6.4 kb	~11.5 kb

Analysis of exon II of bovine lactoferrin from bovine genomic DNA

Specific signals were produced by the hybridisation of exon II sequences from bovine lactoferrin to the genomic DNA (Figure 48). These signals corresponded to fragment sizes predicted from earlier restriction mapping analyses of λOHNZ1 (Section 3.4; Figure 27). There was no correlation between the sizes of fragments which hybridised and those generated from clone I.

Table 23: Hybridisation analysis of exon II sequences from bovine lactoferrin to genomic DNA

Sizes of fragments which hybridised were predicted from earlier restriction mapping analysis of clone I and λOHNZ1 (Sections 3.1 and 3.4)

Restriction endonuclease	Predicted hybridisation fragment sizes (clone I)	Predicted hybridisation fragment size (λOHNZ1)	Observed hybridisation fragment sizes
Bam HI	0.531 kb	1.58 kb	~1.6 kb
Eco RI	2.025 kb	8.8 kb	~8.8 kb

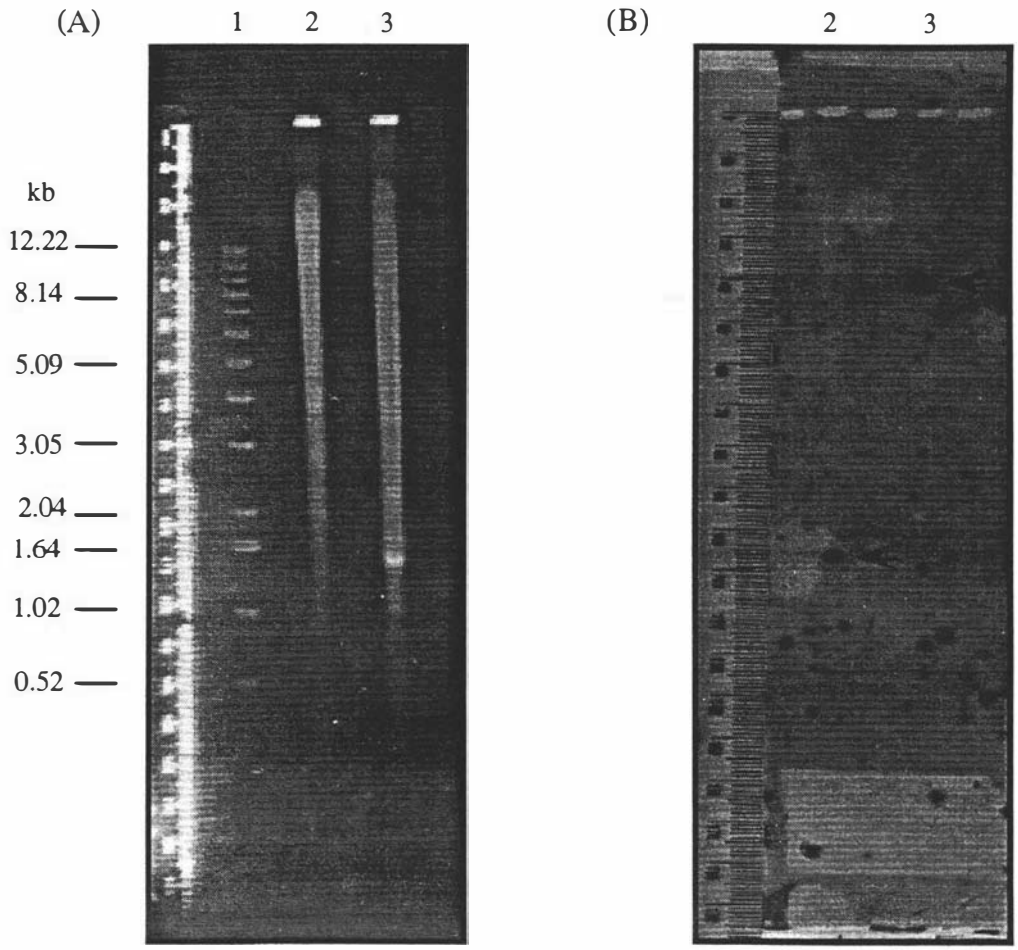


Figure 48: Agarose gel electrophoresis and Southern blot and hybridisation analysis of bovine genomic DNA using a radiolabelled probe derived from exon II sequences of bovine lactoferrin.

(A). Gel photograph of genomic DNA which had been digested to completion.
(B). Autoradiograph of Southern blot hybridsed with a radiolabelled probe derived from exon II. The BRL 1 kb DNA ladder was removed from the agarose gel prior to Southern transfer because it was known to hybridise to probes derived from bovine lactoferrin sequences (See Figure 6). The arrows indicate the fragments which hybridised to the exon II probe.

(1). BRL 1 kb DNA ladder, (2). Genomic DNA (~20 µg) digested with Bam HI,
(3). Genomic DNA (~20 µg) digested with Eco RI

The data produced by Southern blotting of genomic DNA and hybridisation analysis confirmed that the λOHNZ1 clone contained bovine lactoferrin DNA fragments which were analogous to those detected within the bovine genome. These results also indicated that clone I, isolated from the Clonetech library, contained spurious DNA sequences. The observed sequence homology between the two clones, suggested that only 193 bp immediately 5' to exon II contained sequences corresponding to bovine lactoferrin intron I. The 1318 bp of clone I sequence which was not homologous to the sequence in λOHNZ1 was not likely to represent intron I sequences of bovine lactoferrin. Recently the authenticity of some commerical libraries has been questioned (Anderson, 1993; Savakis and Doelz, 1993). Computer analysis of putative human cDNA sequences indicated a high degree of sequence similarities with yeast. Yeast DNA is frequently used as a carrier to increase the efficiency of DNA precipitation. This procedure could cause yeast contamination of DNA libraries. Anderson (1993) cited Clonetech as a supplier of commercial libraries which contained yeast contamination.

The origin of the spurious DNA sequences within clone I was not investigated further. Nevertheless, the presence of these sequences accounted for the non-identical restriction fragment sizes and the sequence discrepancies observed between clone I and λOHNZ1. Clearly, λOHNZ1 contained authentic bovine genomic DNA sequences which were appropiate for this current study.

3.8 Expression of Reporter Gene Constructs

3.8.1 Human Growth Hormone

Four reporter gene constructs were prepared to investigate the functionality of the bovine lactoferrin promoter within primary bovine mammary and COMMA-1D cells.

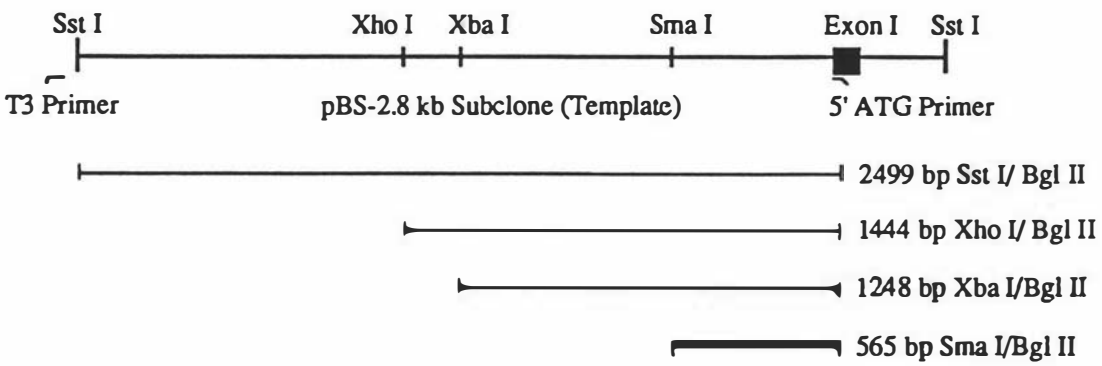
Preparation of Promoter Constructs

The bovine lactoferrin promoter sequences were prepared by PCR using the high fidelity *Pfu* DNA polymerase. An oligonucleotide primer (5' ATG) was designed to remove the bovine lactoferrin translational start site (ATG) as it was critical to obtain translation from the human growth hormone start codon. A T3 oligonucleotide, which binds to the T3 bacteriophage promoter within the pBluescript® vector, and the 5' ATG primer were used to amplify a 2.5 kb fragment from the λOHNZ1 subclone, pBS-2.8

kb. The amplified product contained 2500 bp of the bovine lactoferrin promoter, minus the translational start codon, and all of the Bluescript® plasmid multiple cloning site.

All PCR products were cleaved by the restriction endonuclease Bgl II. This unique restriction site was incorporated into the PCR product by the 5'ATG primer. Cleavage at this site generated a cohesive end which was compatible with the Bam HI restriction site within the pØGH vector. Sst I digestion within the Bluescript® multiple cloning site of the amplified product produced the 2499 bp Sst I/Bgl II insert. The 1444 bp Xho I/Bgl II, 1248 bp Xba I/Bgl II and 565 bp Sma I/Bgl II insert fragments were generated by cleavage within the promoter sequences by the respective restriction endonucleases. These fragments (Figure 49) were all purified from agarose gels and then directionally cloned into the vector pØGH (4817 bp) which lacks an eukaryotic

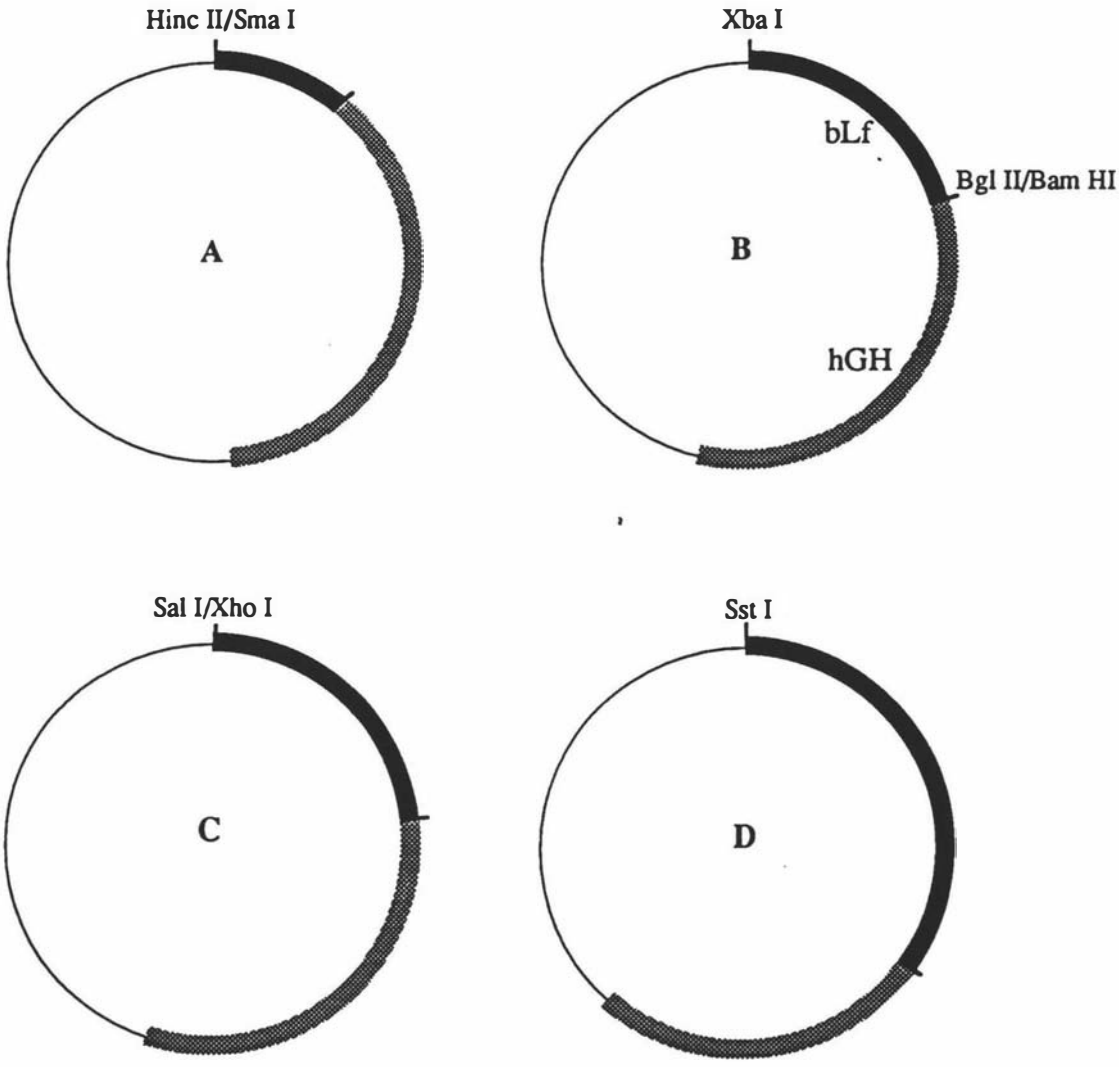
Figure 49: Schematic representation of promoter fragments used to prepare expression constructs



promoter. This vector contains the human growth hormone reporter gene (Seldon *et al.*, 1986). Human growth hormone (hGH) is a secreted 191 amino acid protein which is easily detected and quantitated within spent culture media using an ELISA. The 2499 bp Sst I/Bgl II fragment was ligated into the Sst I/Bam HI restriction sites of pØGH. The compatible Sal I and Xho I restriction sites allowed the cloning of the 1444 bp Xho I/Bgl II insert into the Sal I/Bam HI sites of pØGH. The Xba I/Bgl II and Sma I/Bgl II inserts were ligated directly into the Xba I/Bam HI and Hinc II/Bam HI sites respectively of pØGH. Ligated plasmid DNA was introduced into XL-1 Blue competent cells by heat shock. Plasmid DNA was prepared from single colony transformants and analysed by restriction endonuclease digestion. All promoter constructs (Figure 50) were sequenced to confirm that no errors had been introduced during the production of the clones.

Plasmid DNA representing the isolated clones was prepared on a large scale, precipitated using ethanol and sent to Professor Schanbacher at Ohio State University for analysis within COMMA-1D and primary bovine mammary cells.

Figure 50: pØGH promoter constructs containing the human growth hormone (hGH) reporter gene and various lengths of the bovine lactoferrin (bLf) promoter



A. 565 bp Sma I/ Bgl II fragment of the bovine lactoferrin promoter cloned into the Hinc II/Bam HI restriction sites of pØGH

B. 1220 bp Xba I/Bgl II fragment of the bovine lactoferrin promoter cloned into the Xba I/Bam HI restriction sites of pØGH

C. 1444 bp Xho I/Bgl II fragment of the bovine lactoferrin promoter cloned into the Sal I/Bam HI restriction sites of pØGH

D. 2499 bp Sst I/Bgl II fragment of the bovine lactoferrin promoter cloned into the Sst I/Bam HI restriction sites of pØGH

Expression of Promoter Constructs

The COMMA-1D cells and primary bovine mammary cells were transfected with three of the pØGH expression constructs as described in section 2.2.19. The 565 bp Sma I/Bgl II promoter construct was not analysed for promoter activity. The plasmid pCMV/hGH, which contained the cytomegalovirus promoter coupled to the hGH reporter gene, was used as a positive control. The expression of hGH by this vector was used as an indication that the transfection procedure was functioning and that the cells were capable of secreting hGH. Cells were also transfected with pØGH to monitor background levels of expression.

The constructs which were introduced into the primary bovine mammary cells and COMMA-1D cells produced inconclusive results. No significant difference in levels of secreted hGH were detected between untransfected cells and cells containing the lactoferrin promoter constructs. Similar levels of hGH were obtained for cells transfected with lactoferrin promoter constructs and cells transfected with the control vector pØGH (Table 24). Duplicate samples containing the same construct produced variable results.

In contrast, cells transfected with pCMV/hGH produced distinguishable levels of hGH (Table 24). This suggested that the DNA had been successfully introduced into the cells by the transfection process and that the cells were capable of secreting hGH into the medium. Native bovine lactoferrin secreted by the primary mammary cells was measured by ELISA to indicate the potential of the gland to transcribe the bovine lactoferrin gene. Levels of up to 800-1000 ng/ml were detected in both transfected and non-transfected cells (data not shown). This suggested that the absence of production of hGH by cells transfected with lactoferrin promoter constructs was not due to an inability of the cells to initiate transcription of the bovine lactoferrin gene.

Collectively these results implied that the bovine lactoferrin promoter was inactive within primary bovine mammary and COMMA-1D cells. Liu and Teng (1991) studying the activity of the mouse lactoferrin promoter reported a ~12 fold difference between the activity directed by the lactoferrin promoter and that of the positive control vector pSV-CAT. Based upon this finding, it is possible that the hGH assay was not sensitive enough to detect very low levels of expression directed by the bovine lactoferrin promoter. Seyfert *et al.* (1994) proposed that the lack of sequence motifs such as GATA-1 and an estrogen responsive element which are present in the mouse and human lactoferrin promoters but absent from the bovine lactoferrin promoter, may account for the comparatively low transcription rate of the bovine lactoferrin gene.

Table 24: Secretion of hGH by primary bovine mammary and COMMA-1D cells

Cells in duplicate wells (a & b) were transfected on day 0 with ~1 μg of plasmid DNA complexed with DEAE-dextran, poly-ornithine and poly-glutamate-histamine in the presence of an osmotically balanced, buffered solution. Spent media was collected from each well and stored at minus 80°C until analysed for the presence of secreted hGH by ELISA.

Primary bovine mammary gland cells

Plasmid		Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18	Day 20	Day 22
non-transfected cells	a	0.000	0.860	1.008	1.324	0.000	1.946	1.029	0.000	0.643	0.510	1.547	0.327
	b	0.000	0.993	2.118	1.294	0.393	2.333	1.363	0.000	0.362	0.423	0.847	0.392
pØGH	a	0.000	0.331	1.642	0.419	0.000	0.000	0.000	0.000	0.000	0.532	0.000	0.000
	b	0.000	0.445	1.360	0.838	0.326	0.640	0.604	0.312	0.370	1.110	0.000	0.000
pCMV/hGH	a	0.000	3.066	14.40	56.78	44.28	>140	>140	52.57	68.26	130	49.88	25.00
	b	0.000	2.462	20.80	45.36	52.86	57.79	55.60	56.65	50.69	140	37.69	23.73
1248 bp bLf + pØGH	a	0.000	0.000	0.815	1.589	0.000	0.979	1.342	2.361	0.774	1.105	2.104	1.369
	b	0.000	0.656	1.088	1.309	0.000	0.777	0.880	0.410	0.802	0.205	1.500	3.668
1444 bp bLf + pØGH	a	0.000	0.672	0.723	0.705	0.717	0.852	0.441	0.878	0.873	0.835	1.109	1.237
	b	0.000	0.676	0.556	0.635	0.000	0.488	0.000	0.000	0.699	0.249	0.000	0.864
2499 bp bLf + pØGH	a	0.000	0.402	0.406	0.815	0.301	0.000	0.000	0.844	0.000	0.586	0.000	0.510
	b	0.000	0.719	0.000	0.680	0.000	0.000	0.000	0.000	0.000	0.735	0.000	0.400

COMMA-1D Cells

Plasmid		Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18	Day 20	Day 22
non-transfected cells	a	0.00	1.19	0.34	1.04	1.23	0.49	0.84	0.87	0.00	0.87	0.86	0.00
	b	0.14	0.65	0.58	1.36	0.79	0.00	2.45	0.98	0.00	0.68	0.00	0.00
pØGH	a	0.19	0.72	0.00	0.00	0.51	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	b	0.41	0.33	0.00	1.12	0.67	0.00	0.00	0.00	0.00	0.30	0.00	0.00
pCMV/hGH	a	0.22	14.52	14.21	10.97	22.69	13.81	29.42	7.92	14.90	18.41	16.68	19.10
	b	0.69	14.46	8.13	12.38	48.42	14.80	26.11	26.65	8.72	14.71	20.94	19.12
1248 bp bLf + pØGH	a	0.00	0.00	1.12	0.00	0.00	5.25	0.00	1.55	0.83	1.27	0.00	0.78
	b	0.00	0.20	2.78	0.83	2.46	0.90	0.00	0.70	1.14	1.71	0.00	0.81
1444 bp bLf + pØGH	a	0.39	0.00	1.41	0.49	1.41	1.23	0.00	1.36	1.17	0.31	0.00	1.73
	b	0.00	0.00	1.07	0.41	0.28	1.60	0.00	0.76	0.85	0.00	0.00	0.37
2499 bp bLf + pØGH	a	0.36	1.17	0.53	0.70	1.21	0.00	0.00	0.87	0.29	0.56	0.00	0.29
	b	0.00	0.43	0.52	0.42	0.57	0.00	0.00	0.71	0.00	0.00	0.00	0.00

Theoretically, primary bovine mammary cells offer the most physiological system to investigate the activity of the bovine lactoferrin promoter *in vitro*. These cells should contain all of the transcription factors necessary for expression directed by the bovine lactoferrin promoter. However, long-term culture of lactating mammary cells, in particular bovine mammary cells, is technically demanding. The culture conditions necessary for the correct differentiation of bovine mammary cells are still relatively unknown. The primary bovine mammary cells used in functional assays carried out at Ohio State University, were grown on floating collagen gels. The transfection procedure used for these experiments prevented the primary mammary cells from differentiating (F. Schanbacher, Pers. Comm.). Differentiation involves the formation of organelles such as rough endoplasmic reticulum, mitochondria and golgi; responsiveness to hormones, and the ability to secrete milk proteins. The absence of further differentiation of the transfected primary cells implied that the hormonal or physical environment required for this process to'occur were not optimal. The endogenous levels of bovine lactoferrin were monitored within the primary mammary cell cultures and levels up to 1000 ng/ml were detected in both transfected and untransfected cells. The significance of these values is not known. The primary cells were prepared by short-term enzymatic digestion of bovine peak-lactation mammary tissue. It is possible that the preparation of the epithelial cells stimulated the cells into a non-lactational or involuting state. To date, it is not known whether lactoferrin expression is differentiation-dependent. The levels of endogenous bovine lactoferrin demonstrated that some of the primary bovine cells used within the transfection experiment were capable of synthesising and secreting lactoferrin. It is likely that the primary cells in culture were a mixed population of differentiated and undifferentiated, transfected and untransfected cells. Accordingly, the cells which actually produced the native lactoferrin measured within these experiments could not be determined. Furthermore, it was not possible to relate the level of lactoferrin measured *in vitro* to *in vivo* values as both of these values were expressed as concentrations. The presence of native lactoferrin demonstrated that some cells within this culture system were capable of producing bovine lactoferrin.

It would be optimal to introduce the reporter gene constructs into primary bovine mammary cells which were in an involuting or non-lactational state since the highest levels of lactoferrin are produced during these periods (Schanbacher *et al.*, 1993; Welty *et al.*, 1976). Monitoring the protein levels of both casein and lactoferrin may provide an indication of the lactational status and cellular function of cultured mammary cells. However, it is not currently possible to define the precise lactational status of the cultured mammary cells or to induce differentiation of the cells into specific lactational states.

The plasmid DNA was introduced into both the primary mammary cells and COMMA-1D cells by a transfection process which had been optimised for the activity of the human cytomegalovirus (CMV) promoter. Cytomegalovirus, a member of the herpes virus group, is a common ubiquitous pathogen which infects a wide range of species, including cattle, causing both persistent and latent infections. *In vivo*, a number of cell types including epithelial cells can be infected. The CMV enhancer, which was also present in the control plasmid pCMV/hGH, is a strong transcriptional element consisting of a series of repeated sequence motifs which contain binding sites for cellular transcription factors such as cAMP-responsive element (CRE), nuclear factor κB (NF-κB) and activator protein-1 (AP-1). These features allow the CMV promoter to drive high levels of expression of recombinant proteins in a wide variety of eukaryotic cell types.

It is likely that both the CMV and the bovine lactoferrin promoters exhibited different activities within the experimental system which was used to investigate the functionality of the lactoferrin promoter. pØGH, unlike the control plasmid pCMV/hGH, contained no enhancer element. As a consequence of this, transcription of the hGH reporter gene by any functional promoter inserted into pØGH would occur at a lower level than a construct containing an active enhancer element, as in pCMV/hGH. Accordingly, this prevented direct comparison of the measured hGH levels produced by these promoters, providing no indication of the efficiency of the transfection of the lactoferrin promoter constructs. In addition, the transfection procedure was unlikely to result in maximal bovine lactoferrin promoter activity. Optimising the transfection procedure for activity of the lactoferrin promoter by varying cell numbers, transfection buffer conditions and the amount of plasmid DNA within each transfection, may lead to detectable levels of hGH within the COMMA-1D and primary bovine mammary cells. The inclusion of an enhancer element which was active within the experimental conditions used may also result in elevated lactoferrin promoter activity and possibly would yield detectable levels of hGH within these experiments.

The plasmid pCMV/hGH demonstrated that the mammary cells could secrete hGH, however, this did not demonstrate the ability of the cells to produce hGH from a pØGH vector containing a functional promoter.

COMMA-1D cells are a mouse mammary epithelial cell line which originally exhibited several properties specific for normal mouse mammary gland function (Danielson *et al.*, 1984). However, the COMMA-1D cells used in these investigations were from a late passage (~30) of this cell line. Successive passaging of COMMA-1D cells is known to result in a loss of differentiation potential, i.e., a loss in the ability to form alveolar cells which can synthesis milk proteins during lactation. Consequently, the COMMA-1D cells used in these experiments were likely to represent an

undifferentiated mammary cell system. This may account for the reduced secreted levels of hGH produced by the CMV promoter within the COMMA-1D cells compared with the corresponding values for primary mammary cells as DNA replication and expression requires differentiated cells. The validity of the COMMA-1D transfected cell results was unclear. Firstly, it is not known if lactoferrin expression is differentiation dependent and secondly, it is likely that the COMMA-1D cells used in these experiments did not exhibit normal mouse mammary gland function. The implications of these results, however, are that if the CMV is a stronger promoter of hGH transcription and secretion than there may be insufficient activity from the bovine lactoferrin promoter to detect any transcription directed by this promoter in the current experimental conditions.

Other factors, such as the distance between the lactoferrin TATA box and the translational start point of the hGH gene within the constructs, may influence transcription initiated by the bovine lactoferrin promoter. In the native hGH gene, the distance between the TATA binding site and the translational start codon is 92 bp while for bovine lactoferrin it is 68 bp. The distance between the bovine lactoferrin TATA binding site and the hGH translational start codon was 125 bp in the promoter constructs. The effect on hGH transcription and expression of this distance between these two DNA elements is not known, however it is possible that this distance was suboptimal in these constructs.

In conclusion, the analysis of the bovine lactoferrin promoter within the COMMA-1D and primary mammary cells produced inconclusive results.

3.8.2 Luciferase Reporter Gene Constructs

Since the expression of the bovine lactoferrin promoter-hGH constructs in COMMA-1D and primary bovine mammary cells produced inconclusive results (Section 3.8.1), additional constructs were prepared using the more sensitive Promega luciferase reporter gene system. These reporter vectors contained the coding region for firefly (*Photinus pyralis*) luciferase. The expression of this gene product was used to monitor transcription from the bovine lactoferrin promoter in transfected eukaryotic cells.

Preparation of Constructs

Two constructs containing different lengths of the bovine lactoferrin promoter (Sma I 565 bp & Sst I 2499 bp) were prepared in the plasmid vector pGL-2 Enhancer

(pGL-2E) to investigate the functionality of the bovine lactoferrin promoter (Figure 51). pGL-2E contains an SV40 enhancer element, located downstream of the luciferase gene (*luc*). This element increases the rate of transcription of the *luc* gene so that very low levels of expression can be detected.

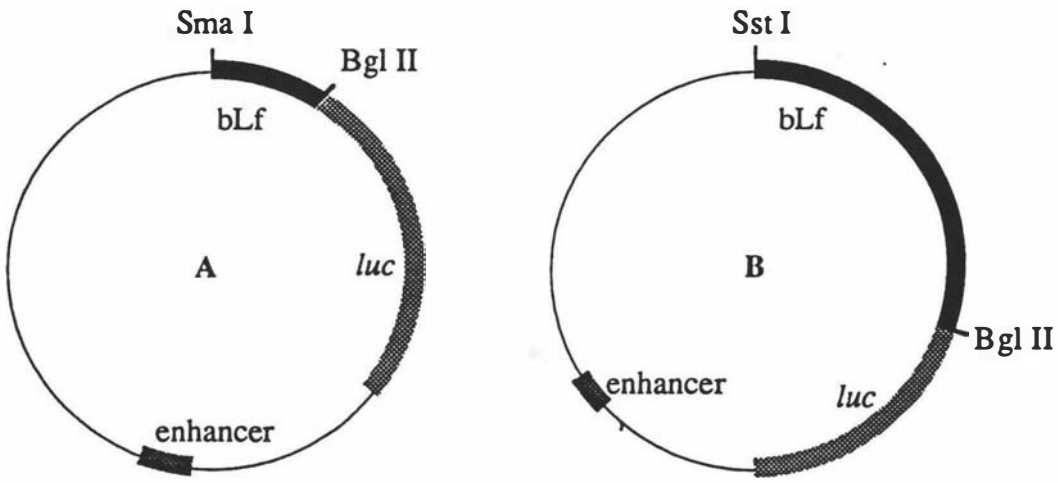


Figure 51: pGL-2E bovine lactoferrin promoter expression constructs

A. 565 bp Sma I/Bgl II bovine lactoferrin (bLf) promoter fragment cloned into pGL-2E vector

B. 2499 bp Sst I/Bgl II bovine lactoferrin promoter fragment cloned into pGL-2E vector

Bovine lactoferrin fragments were prepared by PCR as in section 2.2.17 using the high fidelity *Pfu* polymerase. The amplifed DNA was cleaved with the appropriate restriction enzymes to generate the cloning sites. The 565 bp Sma I/Bgl II and the 2499 bp Sst I/Bgl II lactoferrin promoter fragments were purified from agarose gels and were introduced into the appropriate restriction sites within the pGL-2E vector. Plasmid DNA was introduced into *E.coli* (strain XL-1) by heat shock and transformants were selected at random from colonies grown on LB-amp plates. Plasmid DNA was prepared from single colonies and analysed by restriction endonuclease digestion. A single clone for each construct was selected and plasmid DNA was prepared on a large scale.

Transfection Results

Due to time limitations, the promoter activity of only the 565 bp Sma I pGL-2E construct was investigated by transfecting COS cells with 5, 10 or 20 µg of the plasmid DNA as described in section 2.2.20. All cells were co-transfected with the plasmid vector pCH110 (5 µg). pCH110 contains the functional *lacZ* gene which is translated into the β-galactosidase enzyme. Monitoring transfected cell extracts for β-galactosidase activity allowed transient expression assays to be corrected for variable

cell numbers and variable transfection efficiencies. The cells were harvested ~36 hours after the removal of the transfection solution and the cell extract was assayed for luciferase and β-galactosidase activity (Table 25).

Table 25: Analysis of cell extracts from transfected COS cells

Normalised values were calculated by dividing the measured luciferase activity by the β-galactosidase values. This was performed in an attempt to correct the individual transfections for variable cell numbers and transfection efficiencies.

Transfected DNA		Luciferase Activity	β-Galactosidase Activity	Normalised Values
un-transfected cells		0.000	0.000	
5 µg pCH110		0.063	0.017	3.71
10 µg pGL-2C/pCH110	a	4.657	0.027	172.48
	b	5.762	0.045	128.04
10 µg pGL-2E/pCH110	a	0.144	0.048	3
	b	0.094	0.036	2.61
5 µg pGL-2E Sma/pCH110	a	1.844	0.062	29.74
	b	0.589	0.030	19.63
10 µg pGL-2E Sma/pCH110	a	7.056	0.116	60.83
	b	9.259	0.149	62.14
20 µg pGL2E Sma/pCH110	a	9.814	0.095	103.31
	b	8.878	0.091	97.56

Luciferase activity was detected in cell extracts prepared from cells which had been transfected with the lactoferrin promoter construct. This activity was significantly above background levels which indicated the bovine lactoferrin promoter was active within COS cells. Furthermore, the promoter activity increased with increasing amounts of plasmid DNA in the transfections. Duplicate transfection reactions produced comparable normalised values for all constructs investigated. The normalised values of activity generated by the luciferase control vector (pGL-2C) were at least twice the values observed for the transfection of the same amount of bovine lactoferrin promoter plasmid. This implied that the SV40 promoter and enhancer within the pGL-2C vector were stronger transcriptional elements than the elements present within the first 565 bp of the bovine lactoferrin promoter.

These preliminary results demonstrated that this 565 bp fragment of bovine lactoferrin was a functional promoter, capable of directing the transcription of the luciferase gene within COS cells. COS cells are a fibroblast-like cell line which originate from African green monkey kidney cells and have been transformed with an origin-defective mutant of simian virus 40 (SV40). COS cells are not a physiological system to investigate the expression of bovine lactoferrin since fibroblast cells do not express lactoferrin *in vivo*. However, unlike mammary cells, COS cells are easily cultured *in vitro* and are widely used for eukaryotic expression studies.

COS cells contain the ubiquitous general transcription factors which allow basal promoter activity. However these cells lack the tissue- and species-specific transcription factors which are likely to regulate the expression of the bovine lactoferrin gene *in vivo*. Since the regulation of bovine lactoferrin expression has not yet been characterised, the affect that the absence of these factors would have upon the expression of the lactoferrin promoter can not be determined using this expression system. The absence of specific transcription factors which may down-regulate the bovine lactoferrin promoter *in vivo* may explain the presence of luciferase activity within the COS cells which were transfected with the lactoferrin promoter constructs.

The GeneLight™ reporter system used in these studies contained the coding region of firefly luciferase which was used to monitor transcriptional activity in calcium phosophate transfected COS cells. Both the control plasmid, pGL-2C, and the vector into which the bovine lactoferrin promoter fragments were inserted, pGL-2E, contained an SV40 enhancer located downstream of the luciferase gene. This enhancer element aids the verification of functional promoter elements because it results in elevated transcription of the luciferase gene (*luc*). Comparison of the data obtained from the hGH and luciferase reporter gene investigations suggested that the presence of this enhancer element may have been a significant factor for the detection of transcriptional activity of the bovine lactoferrin 565 bp promoter fragment in the luciferase reporter gene expression system. The presence of the enhancer element in both the lactoferrin promoter constructs and the control plasmid allowed the direct comparison of the luciferase activity produced by these plasmids. From this it could be concluded that the GeneLight™ reporter vectors were being successfully introduced into the COS cells by the calcium phosphate transfection procedure. Furthermore, as both the control plasmid, pGL-2C, and the lactoferrin promoter construct were similar sizes (6046 bp and 6419 bp respectively) and were constructed of similar DNA sequences, it was concluded that these plasmids were likely to be transfected with approximately the same efficiency. Within the mammary cell expression studies It was not possible to speculate on the transfection efficiency of the lactoferrin-hGH constructs when introduced into the mammary cells because the vector in which these were constructed, pØGH, was different from the control plasmid pCMV/hGH. Accordingly, pCMV/hGH could not be considered an appropriate measure of the transfection efficiency within this experiment.

The luciferase promoter constructs and control plasmids were all co-transfected with the β-galactosidase plasmid pCH110. This process is based upon the assumption that both the β-galactosidase plasmid pCH110 and the luciferase plasmids are transfected into the COS cells with the same efficiency and are expressed independently of each other within these cells. While monitoring transfected cell extracts for

β-galactosidase activity enabled variable cell numbers and transfection efficiencies to be taken into account, it is not known if sequences within these individual vectors influenced the expression of the other plasmid within the transient expression system. Consequently, some caution is required when interpreting the luciferase activities measured in these experiments.

A comparison could not be made between theresults of the luciferase expression study and the human growth hormone expression data because the promoter activity of the lactoferrin 565 bp fragment was not investigated in mammary cells. However, the luciferase transient expression system appears to provide a rapid, sensitive, and easily manipulated method for investigating the functionality of the bovine lactoferrin promoter *in vitro*. This expression system can be used to initiate studies directed towards the characterisation of transcriptional elements which participate in the control of expression of the bovine lactoferrin gene. Subsequent analyses in bovine primary mammary cells will allow the identification and isolation of tissue and species-specific elements. These investigations will provide further insights into both the transcriptional regulation of the bovine lactoferrin gene and the biological role of the protein.

3.9 Summary

The regulation of lactoferrin appears to be controlled in a highly specific manner, dependent upon developmental stage, the tissue and the species being investigated. Protein levels of lactoferrin vary dramatically in milk. RNA analyses carried out by Schanbacher *et al.* (1993) suggested that transcriptional regulation may be a significant factor in controlling the expression of bovine lactoferrin in the mammary gland. The primary aim of this project was to isolate genomic regulatory sequences of bovine lactoferrin to enable the characterisation of sequences which were involved in the transcriptional control of bovine lactoferrin expression.

Initially, three clones were isolated from an amplified Clonetech bovine genomic library. Hybridisation analysis suggested that one of these isolates, Clone I, contained sequences which were complementary to a probe containing sequences representing exon I and exon II of bovine lactoferrin . Exon II was located within a 531 bp Bam HI fragment contained within a ~2 kb Eco RI fragment isolated from clone I. This Bam HI fragment was used as a hybridisation probe in later investigations. Exon I sequences of bovine lactoferrin were not detected in any of the clones isolated from the Clonetech genomic library. Further screening of this library did not produce any additional clones.

An unamplified bovine genomic library was subsequently provided by Professor Schanbacher at Ohio State University. Screening of this library with a probe containing sequences representing exon I of bovine lactoferrin resulted in the isolation of the λOHNZ1 clone. This clone was characterised by detailed restriction mapping and was found to contain a ~17.3 kb insert which exhibited complementarity to both exon I and exon II sequences of bovine lactoferrin. Hybridisation analysis of the λOHNZ1 clone indicated that the insert had been cloned in a 3' to 5' orientation relative to the left and right arms of the λFIX II vector, and contained ~10 kb of DNA sequence 5' to exon I.

Hybridisation analyses of the two genomic clones, clone I and the λOHNZ1 clone, indicated that the exon II sequences of bovine lactoferrin were contained within non-identical restriction endonuclease fragments. This discrepancy was investigated initially by restriction analysis of a ~6.4 kb Xba I fragment which was isolated from λOHNZ1 and had been shown to contain exon I and exon II sequences. The results of these investigations were consistent with the mapping analysis of this region of the λOHNZ1 clone, however they did not establish the reason for the observed differences between these two clones. Two fragments containing exon II sequences, ~810 bp Xho

I/Eco RI and ~1.58 kb Bam HI, were isolated from the λOHNZ1 clone and were partially sequenced. Comparison of this sequence data with that obtained from the analysis of a ~2 kb Eco RI fragment isolated from clone I showed that these DNA sequences were divergent in a position 5' to exon II. This suggested that the variation in restriction fragment sizes observed between clone I and λOHNZ1 was likely to be a consequence of DNA sequence heterogeneity. Southern hybridisation analysis of bovine genomic DNA was carried out to determine whether either clone I or λOHNZ1 contained authentic bovine lactoferrin sequences. Genomic DNA, digested to completion, was hybridised to radiolabelled probes derived from the bovine lactoferrin cDNA and which represented exon I and exon II. It was concluded that the λOHNZ1 clone contained DNA sequences which were an accurate representation of the lactoferrin gene within the bovine genome. Clone I was not investigated further as it was concluded that the spurious sequences within this clone accounted for the non-identical restriction fragment sizes and the sequence discrepancies between this clone and λOHNZ1. Consequently, the λOHNZ1 clone was selected for further characterisation.

Restriction mapping analysis of the λOHNZ1 clone and a ~8.6 kb Eco RI fragment isolated from λOHNZ1 indicated that a ~2.85 kb Sst I fragment was likely to contain exon I and the proximal promoter region of the bovine lactoferrin gene. As this region of the bovine lactoferrin gene was the primary focus of this study, the ~2.85 kb Sst I fragment was isolated and characterised by dideoxy sequence analysis. The DNA sequence obtained exhibited high (~99%) sequence homology to a bovine lactoferrin promoter sequence (1007 bp) which was reported during these investigations (Seyfert *et al.*, 1994). A computer sequence homology search indicated that several putative transcription factor binding sites may be located within the sequence of the ~2.85 kb fragment. These preliminary findings require additional analyses to establish the validity of transcription factor binding interactions at these cis-acting DNA sites before the significance of these results can be ascertained.

Two reporter gene systems were used to investigate the functionality of the bovine lactoferrin promoter. The lactoferrin promoter fragments were prepared by PCR which enabled the removal of the lactoferrin translational start codon and the incorporation of a Bgl II restriction site for use in the insertion of these fragments into the reporter gene vectors. Three human growth hormone reporter gene constructs containing different lengths of the lactoferrin promoter were transfected into primary bovine mammary cells and COMMA-1D cells. Human growth hormone could not be detected within the medium collected from cells transfected with the lactoferrin

promoter constructs. Given the complexity of the expression system used for these experiments, the detection method may not have been sufficiently sensitive to detect promoter activity. The activity of an additional lactoferrin promoter construct containing ~565 bp of the 5' flanking region of the bovine lactoferrin coding sequence and the luciferase reporter gene was investigated within COS cells. Significant levels of luciferase activity were detected in the cell extracts prepared from cells transfected with this construct. This demonstrated that this expression plasmid contained a functional promoter and that the primary aim of this investigation had been achieved.

These investigations will enable further studies, directed at elucidating the transcriptional regulation of the bovine lactoferrin gene, to be carried out and will provide insights into the mechanisms controlling the expression of the bovine lactoferrin gene. A detailed knowledge of the mechanisms and factors controlling the expression of lactoferrin within the mammary gland will provide further insights into the biological significance of this protein.

Chapter Four: Future Directions

The successful isolation of the bovine lactoferrin promoter provides a basis for further investigations which will provide an insight into the mechanisms controlling the expression of the bovine lactoferrin gene. This information will not only increase our understanding of the mechanisms involved in the regulation of lactoferrin synthesis but will also provide insights into the molecular mechanisms involved in the expression of milk proteins in general. In addition, this work will complement the knowledge of eukaryotic gene expression and is likely to benefit the production of clinically important proteins within the milk of transgenic animals.

4.1 Determination of the Minimal Promoter of Bovine Lactoferrin

Before extensive characterisation of the bovine lactoferrin promoter can be undertaken, it will be necessary to define the minimal promoter region required for transcriptional activity. This can be achieved by progressively reducing the length of a functional lactoferrin promoter until the ability to support transcription ceases when investigated in a transient reporter gene expression system.

Functional assays involve the introduction of reporter gene constructs, containing the promoter region of interest, into tissue culture cells and monitoring the transfected cells for the expression of the reporter gene. Experiments performed in this study have suggested that monitoring luciferase activity within COS cell extracts may provide a system which will enable an initial analysis of the bovine lactoferrin promoter. However, COS cells do not represent a physiological system for the expression of bovine lactoferrin and consequently may lack some of the transcription factors which bind to the lactoferrin promoter and modulate its' activity *in vivo*. RL 95-2 cells, a human endometrium cell line, have been used successfully to investigate the activity of both the mouse and human lactoferrin promoters (Teng *et al.*, 1992). These cells, which were not available for use in the present study, could be used to assess the ability of the putative bovine lactoferrin promoter to support transcription.

The boundaries of the sequence constituting the minimal functional lactoferrin promoter should be defined for both the 5' and 3' regions. Nucleotides could be deleted from both ends of the promoter region using Bal 31, exonuclease III digestion or PCR. The ~2.5 kb lactoferrin promoter fragment in the pGL-2E reporter vector (figure 51) would be a suitable candidate for Bal 31 or exonuclease III digestion. Bal 31 and exonuclease III digestion both have the advantage that complete sequencing of each deletion mutant produced is not required. In contrast the use of PCR to produce

truncated sequences requires that the entire product must be sequenced to verify the absence of PCR-induced errors. Reporter gene constructs containing various lengths of the mouse lactoferrin promoter were prepared by restriction endonuclease digestion and CAT expression was monitored in transfected RL 95-2 cells (Liu & Teng, 1991). This work demonstrated that the mouse lactoferrin minimal promoter was contained within nucleotides -234 to -21 relative to the start site of transcription. Brunel and co-workers (1988) showed that the 5'-region of the human transferrin gene (nucleotides -119 to -45) was functionally essential for expression of the CAT reporter gene. Deletions which extended into these DNA sequences completely abolished either transferrin or lactoferrin promoter activity in transient reporter gene expression experiments.

The production of transgenic mice containing truncated segments of the lactoferrin promoter, linked to either the CAT or the β-galactosidase reporter genes, could be used to confirm the boundaries of the bovine lactoferrin minimal promoter established within tissue culture cell experiments. The transgenic mice generated for these studies could also be used to identify sequences involved in the tissue- and developmental-specific transcription of the bovine lactoferrin gene. These investigations would establish the bovine lactoferrin DNA sequences necessary for transcriptional activity *in vivo*.

4.2 Binding Sites for Transcription Factors

Promoters are normally composed of clusters of cis-acting DNA sequences which are situated distal and proximal to the transcriptional start point. The binding of trans-acting protein factors to these DNA-motifs and protein-protein interactions between the bound transcription factors forms the transcription initiation complex which facilitates gene expression.

Computer sequence homology searches of the 5'-flanking regions of the mouse lactoferrin, human lactoferrin and human transferrin sequences have identified several putative DNA-binding elements (Liu & Teng, 1991; Teng *et al.*, 1992; Brunel *et al.*, 1988). A preliminary computer sequence homology search has been performed on the bovine lactoferrin promoter region. This has revealed the presence of putative consensus binding elements for several known transcription factors (figure 45). Several techniques, such as DNase I footprinting, electrophoretic mobility shift assays and *in vivo* functional assays, could be used to test the physiological importance of these putative binding sites. Identification of the transcription factor binding sites within the bovine lactoferrin promoter will provide an insight into the factors and possible mechanisms controlling the expression of this gene.

4.2.1 Functional assays

The promoter activities of the mouse lactoferrin and human transferrin genes have been investigated in functional assays by monitoring the CAT enzymatic activity generated by a series of CAT reporter gene plasmids which contained various lengths of the 5'-promoter sequences of these genes (Liu & Teng, 1991; Schaeffer *et al.*, 1989). Variations in the level of CAT activity were detected within the cells transfected with the individual constructs. This was dependent upon the length of the 5'-flanking sequences contained in the reporter constructs. Using this analysis a combination of negative- and positive-acting elements which modulate the expression of the mouse lactoferrin and human transferrin genes *in vivo* were identified.

Regions of functional importance in the bovine lactoferrin promoter could be identified by an experimental approach similar to that used for the mouse lactoferrin and human transferrin regulatory regions as described above. Comparison of the relative luciferase activities in RL 95-2 cells transfected with reporter gene constructs containing different lengths of the bovine lactoferrin promoter would provide an indication of the regions which are important in the transcriptional regulation of the bovine lactoferrin gene *in vivo*. Once these regions have been identified, the ability of specific transcription factors to interact at these sites and modulate bovine lactoferrin gene expression could be investigated in two ways. Firstly, promoter reporter gene constructs containing the minimal bovine lactoferrin promoter could be analysed by the co-transfection with a cloned transcription factor into the expression system. Secondly, specific DNA recognition sequences isolated from the bovine lactoferrin promoter could be further characterised by investigating the ability of these sequences to modulate the transcription from a heterologous promoter in co-transfection studies with cloned transcription factors. These functional analyses are the focus of the following sections.

Co-transfection of transcription factors

RL 95-2 cells are an established tissue culture cell line which have been passaged numerous times. Successive passaging of cells *in vitro* can produce cells which do not exhibit the same morphology and functionality of the cells from which they were originally derived. In particular the expression of tissue and development specific transcription factors may be lost during passage of the cells. Consequently, it can be useful to introduce cloned transcription factors by co-transfection into the cell culture system. Supplementation of the culture media with the appropriate activator ligands, such as steroid hormone, may also be required to enable these transcription factors to be functional within the expression system.

In vivo, murine lactoferrin has been shown to be regulated at the transcriptional level by estrogen (Pentecost & Teng, 1987; Teng *et al.*, 1989). Minimal estrogen

responsiveness of the CAT reporter gene, linked to putative binding sequences for a mouse lactoferrin estrogen receptor, was detected in RL 95-2 cells in the absence of co-transfection of an estrogen receptor expression plasmid. Estrogen stimulation was observed in these cells when co-transfected with an estrogen receptor expression plasmid and growth media supplemented with diethylstilbestrol (Liu & Teng, 1991).

A computer sequence homology search has indicated that the progesterone receptor may interact with the bovine lactoferrin promoter (figure 45). Cotransfection of COS or RL 95-2 cells with a progesterone receptor expression plasmid and lactoferrin promoter reporter gene constructs, in the presence of progesterone, will allow the assessment of the effect of this transcription factor on promoter activity. An increase or decrease in transcriptional activity of the bovine lactoferrin promoter would suggest that the progesterone receptor was able to modulate the expression of the lactoferrin gene. Supporting evidence gained from *in vitro* binding assays (Sections 4.2.2 & 4.2.3) would be critical for these investigations.

Functional analysis of isolated sequences

The ability of isolated DNA-binding sites to modulate transcriptional activity could be investigated in functional assays in tissue culture cells. For example, Liu and Teng (1992) investigated the ability of mouse lactoferrin DNA sequences to confer estrogen inducibility to CAT reporter gene plasmids containing either the heterologous simian virus 40 (SV40) promoter sequences, or the homologous mouse lactoferrin minimal promoter sequences. In these studies the putative estrogen responsive module, positioned 5' to the promoter element linked to the CAT reporter gene, was introduced with an estrogen receptor expression plasmid into cultured RL 95-2 cells. Monitoring the transcriptional activity of the basal and estrogen-induced promoters in this expression system indicated that this sequence element was able to confer estrogen-stimulated transcription to both homologous and heterologous promoters. Reporter gene plasmids containing tandem repeats of the synthetic oligonucleotide representing the estrogen responsive module generated higher levels of expression than a single copy of the oligonucleotide. Furthermore, the introduction of specific point mutations into this sequence element abolished estrogen stimulation. Using these functional analyses, key DNA-binding sites which modulate transcriptional activity can be characterised to provide an indication of their functional significance.

Functionally important regions which modulate transcription of the bovine lactoferrin gene could be characterised by methods similar to those descibed above. The ability of putative regulatory sequences to confer functionality to heterologous and homologous minimal promoter regions within transient transfection experiments would allow regions of transcriptional importance to be identified. Site-directed mutagenesis of these DNA sequences would enable specific residues necessary for binding

interactions to be defined.

The expression of lactoferrin in the mammary gland appears to occur in a highly specific manner, dependent upon the developmental and lactational stage of the gland. Accordingly, it is likely that the bovine lactoferrin promoter will contain DNA sequences which regulate expression of the bovine lactoferrin gene in a tissue- and developmental-specific manner. Recognition sequences for a mammary gland-specific factor have been identified in the gene encoding β-casein (Schmitt-Ney *et al.*, 1992). No homology was detected between the consensus recognition sequence of this factor and the sequence of the bovine lactoferrin promoter when investigated using the VAX GCG "Findpatterns" computer programme.

In vitro and functional assay analyses will provide insights into the factors and mechanisms regulating the transcription of the bovine lactoferrin gene, however the information obtained from these methods may not necessary reflect the *in vivo* regulation of expression of this gene. Accordingly DNA sequence elements responsible for modifying expression of the bovine lactoferrin gene in a tissue-specific and developmental-specific manner *in vivo* would need to be identified using transgenic mice. Adrian *et al.* (1990) used transgenic mice to characterise human transferrin gene sequences which responded *in vivo* to cellular signals affecting expression in various tissues and also to iron administration. Transgenes composed of the CAT reporter gene and truncated segments of the human transferrin 5' regulatory region were introduced into the germline of mice. Expression of the CAT gene in these transgenic mice suggested that the human transferrin sequences located between nucleotides -622 bp and +46 bp were adequate for tissue-specific and iron-responsive expression. Similarly, tissue-specific expression of the bovine lactoferrin gene could be investigated by the production of transgenic mice containing truncated lengths of the bovine lactoferrin promoter linked to a reporter gene. The analysis of RNA samples prepared from various tissues of these transgenic mice would determine the presence or absence of expression.

Primary bovine mammary cells

Primary bovine mammary cells offer the most physiological system for *in vitro* analysis of the bovine lactoferrin promoter. These cells contain all the transcription factors necessary for expression directed by the bovine lactoferrin promoter and consequently should not require the co-transfection of cloned transcription factors. Mammary cells are extremely difficult to culture *in vitro* such that they maintain their normal mammary biochemistry and physiology. Nevertheless, it will be important to confirm the results of functional assays carried out in transformed cells using a primary bovine mammary cell system. Currently, primary bovine mammary cells which

express milk proteins can be cultured for up to 22 days (Talhouk *et al.*, 1993). The experimental conditions required for cell growth and differentiation remain to be elucidated fully. The complexity of the bovine mammary cell expression system will require some prior knowledge of the factors influencing bovine lactoferrin expression. It has been established that factors, such as progesterone, are responsible for alveolar development *in vivo* (Schmidt, 1971). Accordingly, supplementing culture media with these factors may influence the expression of bovine lactoferrin and the functionality of the cells within the expression system. Preliminary investigations of transcriptional activity of the bovine lactoferrin promoter within COS cells and RL 95-2 cells should assist future investigations using primary mammary cells. Recently, primary mammary cells have been transfected with simian virus 40 (F. Schanbacher pers. comm.) creating a bovine mammary cell line which can be passaged *in vitro*. This cell line should prove invaluable in the future study of the transcriptional regulation of the bovine lactoferrin promoter.

The investigation of the transcriptional activity of the lactoferrin promoter in primary bovine mammary cells in these studies was carried out at Ohio State University. Consequently, a bovine mammary cell culture system would need to be established at Massey University for these cells to be used in the investigation of the molecular mechanisms and factors regulating the transcription of the bovine lactoferrin gene. The synthesis of alpha-$_{S1}$casein, a protein of peak lactation milk, can be used as a marker of cell differentiation which is consistent with a lactational state of the gland *in vivo*. Methods used to characterise the functionality of the cells in culture, such as an ELISA to measure the endogenous alpha-$_{S1}$casein and lactoferrin levels synthesised by these cells would also need to be developed.

4.2.2 DNase I footprinting

DNase I footprinting allows the identification of short sequences representing protein-binding sites to be identified within a relatively large DNA fragment. A double stranded fragment of the promoter region which is radiolabelled at one end, is mixed with either purified protein factors or crude nuclear extract. Protein bound to the DNA will protect specific regions from enzymatic attack by DNase I. Maxam and Gilbert sequencing reactions of the same DNA fragment are separated by denaturing polyacrylamide gel electrophoresis alongside the DNase I cleavage products to allow the specific nucleotides involved in the protein interaction to be determined.

DNase I footprinting has been used to characterise the nature of the estrogen receptor and COUP-transcription factor binding interactions with the mouse lactoferrin gene (Liu *et al.*, 1993). Comparison of the individual footprints produced by these proteins bound to the mouse lactoferrin promoter provided data which supported the finding that these binding sites overlapped. Additional footprinting analyses suggested

that these two transcription factors competed for binding to the mouse lactoferrin promoter, providing an indication of a possible transcriptional control mechanism of the mouse lactoferrin gene (Liu *et al.*, 1993).

Seven putative NF-IL 6 binding sites have been identified at various positions within the bovine lactoferrin promoter sequence (Figure 45). Interleukin 6 (IL-6) is a potent mediator of the acute-phase response which occurs following injury or infection (Gauldie *et al.*, 1987). Bovine lactoferrin levels have been shown to increase in response to intramammary infection (Harmon *et al.*, 1976). In addition, it has been postulated that lactoferrin may play a role in inflammation by modulating the levels of key cytokines (Crouch *et al.*, 1992; Machnicki *et al.*, 1993). Consequently, the binding of NF-IL 6 to the lactoferrin promoter may activate the expression of the lactoferrin gene in response to an acute-phase reaction. *In vitro* binding assays, such as DNase I footprinting and gel mobility shift assays, can be used to investigate each of these putative binding interactions.

Initially it will be important to demonstrate that the bovine lactoferrin promoter is actually responsive to IL-6. The IL-6 responsiveness of the promoter could be assessed by functional assays in RL 95-2 cells supplemented with IL-6 at physiological levels. If the promoter is responsive in these studies the putative NF-IL 6 binding sites could then be analysed by DNase I footprinting analysis. In these investigations purified NF-IL 6 and radiolabelled fragments of the lactoferrin promoter (~500 bp) would be mixed and subjected to DNase I cleavage to determine if NF-IL 6 specifically interacts with any of the seven sites identified. The use of purified NF-IL 6 would isolate this binding site from other transcription factors which may be located within the same region of the promoter. These initial DNase I footprinting analyses would provide an indication as to whether NF-IL 6 interacts at specific sites within the bovine lactoferrin promoter. Putative NF-IL 6 DNA-binding sites identified by these studies could be further investigated by analysing the ability of promoter fragments (~250 bp) to form a DNase I footprint with nuclear extracts prepared from bovine mammary glands at various stages of lactation and involution. This work may highlight putative developmental or stage specific interactions. Consensus NF-IL 6 recognition sequences which were protected from DNase I digestion could be further characterised by electrophoretic mobility shift assays and functional assays using co-transfection with the cloned NF-IL6 transcription factor. The cumulative data obtained from all of these analyses should indicate the significance of these putative NF-IL 6 binding sites which were identified by the computer sequence homology search.

4.2.3 Electrophoretic mobility shift assays (EMSA)

EMSA provides a method for investigating the ability of protein factors, either

purified or in a crude nuclear extract, to specifically interact with a radiolabelled, double stranded oligonucleotide probe. The probe generally consists of a short DNA sequence (20-30 bp) which represents a putative binding site for a transcription factor. Protein factors bound to the radiolabelled oligonucleotide cause it to migrate more slowly than the free probe when subjected to electrophoresis through a non-denaturing polyacrylamide gel. This results in the appearance of a retarded band upon autoradiography allowing the presence of bound protein factors to be detected. The specificity of protein-DNA interactions can be tested by the use of competitor oligonucleotides representing specific and non-specific DNA sequences within the promoter region being investigated.

Sequence analysis of the 5'-flanking region of the mouse lactoferrin gene indicated that a putative estrogen-responsive element (ERE) overlapped with a chicken ovalbumin upstream promoter (COUP) element (Liu & Teng, 1991). Liu and Teng (1992) used EMSA to examine the specific interactions between mouse uterine proteins and a synthetic oligonucleotide representing the mouse lactoferrin COUP/ERE element. Specific protein-DNA complexes were observed with whole cell protein extracts prepared from RL 95-2 cells transfected with an estrogen receptor expression plasmid, and nuclear protein extracts prepared from RL 95-2 cells containing the COUP-transcription factor. Addition of excess unlabelled COUP/ERE oligonucleotide abolished the protein-DNA interactions. Antibodies specific to the estrogen receptor and COUP-transcription factors caused an additional retardation, or supershift, of the radiolabelled oligonucleotide bound to the transcription factors, indicating specific protein-DNA interactions.

Protein-binding sites identified by DNase I footprinting analyses of the bovine lactoferrin promoter could be further characterised by EMSA. Comparison of the specific binding interactions obtained from EMSA analyses using purified transcription factors with those obtained using nuclear protein extracts prepared from bovine mammary cells, may provide an indication of the complexity of the protein-binding interactions at these putative regulatory sites. Protein factors binding to the same oligonucleotide sequence as homodimers or heterodimers could be identified by EMSA investigations involving increasing amounts of purified protein factors. Using these methods, proteins-interactions which occur at individual, overlapping or identical DNA-elements may be identified. Identification of these protein factors may provide insights into the mechanisms regulating bovine lactoferrin gene expression.

Once specific binding sites or consensus sequences have been identified methylation interference studies could be used to further characterise DNA sequences which are binding to transcription factors. Methylation of specific guanine residues by dimethyl sulphate inhibits the binding of a transcription factor to that nucleotide. Accordingly, the inability of a particular protein factor to bind to an oligonucleotide

probe with methylated guanine residues in EMSA analyses, allows the identification of key residues involved in protein binding. Methylation interference studies were used by Liu and co-workers (1993) to identify the specific guanine residues in the mouse lactoferrin estrogen response module which were essential for the binding of the estrogen receptor and the COUP-transcription factor. Similar studies could be used for bovine lactoferrin once specific binding of transcription factors has been established.

If specific binding sites for transcription factors can be identified in the bovine lactoferrin promoter, it will be important to identify these proteins. Consensus binding sequences have been identified for many transcription factors (Faisst & Meyer, 1992). It will be important to compare these with any sites identified by DNase I footprinting or EMSA. Authenticity of putative binding sites in the bovine lactoferrin promoter will need to be demonstrated. Purified transcription factors (some of which are available commercially) or enriched cell extracts, prepared from transfection of COS cells with expression vectors containing cDNA sequences of transcription factors, will be useful in these studies. Competition studies between binding sites from the lactoferrin promoter and increasing concentrations of an oligonucleotide representing the consensus recognition sequence will indicate both the specificity and the relative binding affinities of the protein factor at a specific site.

Collectively, the information obtained from analysis of the bovine lactoferrin promoter sequences within functional assays, EMSA and DNase I footprinting procedures will provide an indication of the transcription factors and mechanisms regulating the temporal expression of this gene.

It is not known if *in vitro* footprinting results truly represent the binding of cellular proteins to genomic DNA within the intact cell. Consequently, *in vivo* footprinting has been developed which allows the mapping of binding sites of nonhistone proteins to DNA within the nucleus. This method is based upon the treatment of cells with dimethyl sulphate (DMS) which can freely and rapidly permeate intact cell membranes and cause limited methylation of guanine residues at the N7 position. Methylated DNA isolated from these cells is treated with piperidine which generates a population of DNA fragments of variable lengths due to selective cleavage at methylated guanine residues. Ligation-mediated PCR (Mueller & Wold, 1989) could then be used to produce a sequence ladder representing the cleavage products produced by this procedure. This technique involves the ligation of a common linker to the ends of the DNA generated by piperidine cleavage and amplification of the DNA by the polymerase chain reaction. Primer extension with a specific radiolabelled primer allows visualisation of the amplified cleavage products by autoradiography. Comparison of the cellular DNA which is complexed with proteins, to genomic DNA

which was isolated from the cell prior to methylation (naked DNA) allows nuclease-resistant regions, or DNA footprints, to be identified. This method of *in vivo* footprinting requires greater than $3x10^5$ cells, derived from the same cell population, to generate reproducible results which are not obscured by background signals from physiologically distinct nuclei. Primary bovine mammary cells grown in culture would provide an appropriate cell population for the *in vivo* footprinting analysis of the bovine lactoferrin gene. Comparison of the results obtained from *in vivo* and *in vitro* footprinting experiments enables authentic transcription factor binding sites to be identified within genomic DNA.

4.5 Lactoferrin as a Transcription Factor

It has recently been reported that human lactoferrin interacts with DNA sequences intracellularly causing transcriptional activation of other genes (He & Furmanski, 1995). These investigators proposed that lactoferrin, released by neutrophils in response to infection, sequestered iron, and was taken up by target cells, such as natural killer cells or macrophages, and influenced the transcription of genes by acting as a transcription factor. Lactoferrin has been demonstrated to bind many biological molecules and surfaces as a result of its basic pI (Brock, 1985). Accordingly it is important to establish whether lactoferrin can interact specifically or non-specifically with this putative consensus binding sequence. He and Furmanski (1995) investigated the specificity of the lactoferrin interactions using EMSA, DNase I footprinting and CAT reporter gene expression experiments containing multimers of the putative 11 bp recognition sequence. Competitive binding assays containing increasing amounts of the putative consensus binding sequence were not investigated by these researchers. These experiments are necessary to demonstrate that the lactoferrin molecule is interacting specifically with the consensus binding site and is not demonstrating non-specific interactions.

These experiments do raise an interesting concept whereby lactoferrin may act as a regulator of it's own expression. Repeating these experiments with purified bovine lactoferrin protein and the bovine lactoferrin promoter linked to a reporter gene may provide an insight into the feasibility of this proposal.

Expression of the bovine lactoferrin gene is likely to be differentially regulated at a variety of levels. Using the experimental procedures discussed within this chapter, bovine lactoferrin promoter sequences involved in protein-binding interactions and intracellular factors which modulate the transcription of the bovine lactoferrin gene will be identified. These investigations will provide an understanding of the possible mechanisms involved in the transcriptional regulation of bovine lactoferrin and will help identify the functional significance of this protein.

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4.6 Regulation of Bovine Lactoferrin

Many mechanisms of regulation of expression of the bovine lactoferrin gene are likely to exist. Preliminary investigations by Schanbacher and co-workers have indicated that mRNA stability may be a key factor regulating the expression of the lactoferrin gene (F. Schanbacher, Pers. Comm.). Further investigations could be directed towards integrating the deduced transcription control mechanisms with other cellular processes which influence the overall expression of the bovine lactoferrin gene. A knowledge of the intracellular factors and mechanisms regulating the expression of the bovine lactoferrin gene will help decipher the functional significance of this protein.

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Appendix 1: Nucleotide sequence of an Eco RI fragment isolated from clone I.

Comparison with the bovine lactoferrin cDNA sequence showed this fragment contained 1511 bp of intron I, exon II (uppercase letters), and 350 bp of intron II.

```
      gaattcaggagagcgacgccaccgaattaatttatactgaagctaaccacactgctgttt
    1 ---------+---------+---------+---------+---------+---------+ 60

      aattgagaccatttgaagtaattaatttttagagagataaggttctctccctctgcaagc
   61 ---------+---------+---------+---------+---------+---------+ 120

      atctcacggagggtacgggacgaggcagcagcacagacgtggcagcagggagcctgctca
  121 ---------+---------+---------+---------+---------+---------+ 180

      gggggccagcctgctgccaccagcccgggtggagcccagtgccacagacaggcactgct
  181 ---------+---------+---------+---------+---------+---------+ 240

      tccatacaaggaggagtcaccgagcagacgggagacaagcagagcctgaagaaaatatca
  241 ---------+---------+---------+---------+---------+---------+ 300

      aaagtgccaacaaggaccaaacttgagcattccaagggacctccgatcacaccagtctga
  301 ---------+---------+---------+---------+---------+---------+ 360

      ctgtccgatctcaggtgaagattcaggagggcagccagcattgctccaaacgttgcgctg
  361 ---------+---------+---------+---------+---------+---------+ 420

      tgagaggctaaagaaataaaccaagactgcagaggggaagcagctaagactgaagacggc
  421 ---------+---------+---------+---------+---------+---------+ 480

      gacttcaggacacaccatgcagttctccacatcgacgacagggcttctgcgtgagtcgag
  481 ---------+---------+---------+---------+---------+---------+ 540

      agccaggacggcaggacagtgatgaccatgaatcgtgacacccttggaccgcctcccagg
  541 ---------+---------+---------+---------+---------+---------+ 600

      gccagaaccttacgcagcctccacgacggtcatctgaacacggcactctggtcctgtcca
  601 ---------+---------+---------+---------+---------+---------+ 660

      ttttacagcagggaaagtgcgtgtgttagtagcttagtcgtgtccgactctgtgacccca
  661 ---------+---------+---------+---------+---------+---------+ 720

      tggactgtagtctgccaggctcctcctctatccatagaatcctccaggcaagaatactag
  721 ---------+---------+---------+---------+---------+---------+ 780

      agtgggtggccattcccttctccaggggatcctcccagcccagggattgaacccaggtct
  781 ---------+---------+---------+---------+---------+---------+ 840

      cctgcattgcagcagattatttactgtctgagccaccagggaagcccagcaaagacacgg
  841 ---------+---------+---------+---------+---------+---------+ 900

      gctatggcagggaaagcaaggcttaaacgaggttattcaaactacggtagccccacagcg
  901 ---------+---------+---------+---------+---------+---------+ 960

      agaacccagagtcctagactcagttgattcccaagaccatttccctcacagtgaagccag
  961 ---------+---------+---------+---------+---------+---------+ 1020

      gccaccctccccgaccaggtccaaggaggactgtcctcctagaggcatgcgtcaacatcc
 1021 ---------+---------+---------+---------+---------+---------+ 1080

      caaagtactagtggtttaaggggtcagagccagagccccacacgggcagaccgcgtttca
 1081 ---------+---------+---------+---------+---------+---------+ 1140
```

```
     tcacactcggcaggcatgctggtttttaccaccccaaggtttgtgacaaccctggatcca
1141 ---------+---------+---------+---------+---------+---------+ 1200

     gcaagtttatttggccccgtttttccaacagcactcgctcactcggtgtctctgggtcac
1201 ---------+---------+---------+---------+---------+---------+ 1260

     gttttgataattttcacgttatttccagcttcttcattgctatatttgtggtggtgatgt
1261 ---------+---------+---------+---------+---------+---------+ 1320

     gatcatgtcctcaaacagccctctggccagcatggagtggtcattaggctagcgaaacac
1321 ---------+---------+---------+---------+---------+---------+ 1380

     ctccatcagaggagagtggcctgactcagccacagaatggagagggaaggggagggcccc
1381 ---------+---------+---------+---------+---------+---------+ 1440

     tgcttgagagggcaagccgtctcttccacggacagtgatagagccctcactctttggcct
1441 ---------+---------+---------+---------+---------+---------+ 1500

     ctttctcccagGACTGTGTCTGGCTGCCCCGAGGAAAAACGTTCGATGGTGTACCATCTC
1501 ---------+---------+---------+---------+---------+---------+ 1560

     CCAACCTGAGTGGTTCAAATGCCGCCGATGGCAGTGGAGGATGAAGAAGCTGGGTGCTCC
1561 ---------+---------+---------+---------+---------+---------+ 1620

     CTCTATCACCTGTGTGAGGAGGGCCTTTGCCTTGGAATGTATCCGGGCCATCGCGGtgag
1621 ---------+---------+---------+---------+---------+---------+ 1680

     tccaggccgtaggctgggtgggaccagactgaaagggaagggatccaagtgccatggaaa
1681 ---------+---------+---------+---------+---------+---------+ 1740

     atgtgctcgccctgccccctctgctcttcttcccgagcgcctgtaagttgggagctgtc
1741 ---------+---------+---------+---------+---------+---------+ 1800

     ctctctcctaggacacagcgttattttggaagcagacattgccattctaacatttgaaaa
1801 ---------+---------+---------+---------+---------+---------+ 1860

     ccatctgtagggaaactggcatcaccacacaaattgagaataatgacggtgcaacgatgg
1861 ---------+---------+---------+---------+---------+---------+ 1920

     tggctcagatggtaaagaatccacctgcaatgcaggacacctgggttcgatccctgggtt
1921 ---------+---------+---------+---------+---------+---------+ 1980

     gggaagatcccctggagaaggaaatggcaatgcactccagaattc
1981 ---------+---------+---------+---------+----- 2025
```

Appendix 2: Alignment of exon II bovine lactoferrin sequences isolated from genomic and cDNA libraries.

The cDNA (nucleotides 75-253) sequence of bovine lactoferrin corresponding to exon II was compared with the DNA sequence (nucleotides 1503-1682) obtained from a 2 kb Eco RI fragment derived from clone I. Comparison of the exon II regions illustrated a single base pair substitution (C-T) and 98% sequence homology. Underlined regions represent intron-exon splice junctions.

```
  75 GAGCCCTTGGACTGTGTCTGGCTGCCCCGAGGAAAAACGTTCGATGGTGT 122
       ||  ||||||||||||||||||||||||||||||||||||||||||||
1503 TTCTCCCAGGACTGTGTCTGGCTGCCCCGAGGAAAAACGTTCGATGGTGT 1552

 123 ACCATCTCCCAACCCGAGTGGTTCAAATGCCGCCGATGGCAGTGGAGGAT 172
     |||||||||||||| ||||||||||||||||||||||||||||||||||
1553 ACCATCTCCCAACCTGAGTGGTTCAAATGCCGCCGATGGCAGTGGAGGAT 1602

 173 GAAGAAGCTGGGTGCTCCCTCTATCACCTGTGTGAGGAGGGCCTTTGCCT 222
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1603 GAAGAAGCTGGGTGCTCCCTCTATCACCTGTGTGAGGAGGGCCTTTGCCT 1652

 223 TGGAATGTATCCGGGCCATCGCGGAGAAAAA 253
     |||||||||||||||||||||| ||
1653 TGGAATGTATCCGGGCCATCGCGGTGAGTCC 1682
```

Appendix 3: Restriction endonuclease mapping of λOHNZ1

The λOHNZ1 clone was characterised by restriction endonuclease mapping. The data (restriction digestions and the Southern hybridisation blots) used to deduce the map of λOHNZ1 are shown in section 3.4. A summary of this information is also given in section 3.4. This appendix outlines in detail the full analysis of the λOHNZ1 restriction digestions and the corresponding Southern hybridisation autoradiographs. A map of the λFIX II cloning vector is shown in figure 9. This shows Not I, Sst I and Xba I restriction sites flank the cloning junctions of the insert . Not I, a rare cutting restriction enzyme, cleaved the entire insert from the vector.

A3.1 Analysis of figure 10 (Page 42)

The digestion profile of λOHNZ1 cut with a variety of restriction enzymes is shown in figure 10A. Apparent variation in fragment sizes between digests may be due to slightly different mobilities of fragments across the agarose gel. In the case of enzymes which cleave at the cloning junction, such as Not I, Sst I and Xba I, it was assumed that the λFIX II arms display the correct mobilities of 20 kb and 9.1 kb for the left and right arms respectively. Similarly, a value of ~19.3 kb was assumed for the fragment resulting from Sma I cleavage within the left arm. Variations from these values have been noted within the discussions of each gel and have been used as an indicator of anomalous gel mobility which may lead to errors within the restriction map of λOHNZ1.

The uncut λOHNZ1 DNA in figure 10A, lane 2, was visible as a broad band with slight smearing on the gel. In all lanes representing digestions, the sharp fragment with the highest molecular size was the left arm (~20 kb) plus part of the insert if the restriction enzyme used did not cleave at the cloning site. The faint fragment directly above this represented uncut λOHNZ1 DNA or partial digestion products of a higher molecular size. The insert could be identified by comparison of the Not I and Not I/Eco RI digests. The ~17.3 kb Not I fragment was cleaved in the Not I/Eco RI digestion. Since Eco RI did not cut within the arms of the vector, this fragment was concluded to represent the insert. The other two fragments, ~20 kb and ~9.1 kb, were the λFIX II vector arms. Fragments of ~8.8, 4.17 and 2.0 kb were common to both the Eco RI and Not I/Eco RI digestions indicating that these fragments were produced by Eco RI digestion. A partial digestion fragment of ~6.2 kb in the Eco RI digest implied that the ~4.17 kb and ~2.0 kb fragments were adjacent to each other within the insert. There was insufficient data to assign the location of these fragments within the clone at this time. Two fragments, ~1.2 kb and ~0.98 kb, were unique to the Not I/Eco RI digest and therefore were products of the digestion involving both enzymes. The ~10.7 kb Eco RI fragment was cleaved into two

fragments by Not I; the right arm (~9.1 kb) and one of the ~1.2 kb or ~0.98 kb fragments. The ~9.0 kb Not I/Eco RI fragment was broader and of darker intensity than the corresponding fragment in the Not I digest. This indicated that the two fragments, ~9.1 kb and ~8.8 kb, migrated together during electrophoresis. Theoretically, the sum of the fragment sizes produced by a digestion should be equal to the size of the fragment cleaved. However, the size of the fragments produced by Not I digestion of the ~10.7 kb Eco RI fragment totalled a maximum of 10.3 kb (9.1 + 1.2 kb). The most probable source of error for this discrepancy was in the size determination of the 10.7 kb fragment as this region of the mobility graph was not as accurate as for lower molecular size ranges. Consequently the Eco RI restriction site could not be located more than ~1.2 kb from the Not I site. Accordingly, fragment sizes of ~21 kb and ~10.3 kb (Table 4) were used in conjunction with the other Eco RI digest products to determine a total molecular size of 46.27 kb for the Eco RI cleavage of λOHNZ1. The other Not I/Eco RI fragment, either ~1.2 kb or ~0.98 kb, was produced by Not I cleavage of the left arm (~20 kb). However, agarose gel electrophoresis is not effective at resolving size differences within this molecular size range. Consequently, it was not possible to assign positions to the ~1.2 kb or ~0.98 kb fragments.

λOHNZ1 was cleaved by Sma I to produce six fragments; ~19.3, 9.8, 6.5, 5.7, 3.0 and 2.15 kb. The ~19.3 kb and ~5.7 kb fragments resulted from cleavage within the arms of λFIX II at ~19.3 kb and ~3.4 kb from the cloning junction at the right arm (Figure 11). Double digestion with Not I and Sma I indicated that the ~9.8 kb and ~6.5 kb Sma I fragments were cleaved by Not I. Because Sma I cleaved within the arms of the vector, Sma I fragments which were cleaved by Not I contained the cloning junctions and a portion of the vector. Based upon the position of the Sma I restriction sites relative to the Not I sites within the λFIX II vector (Figure 9), it was concluded that the ~9.8 kb Sma I fragment lay next to the Sma I site within the right arm, as cleavage of this fragment by Not I produced ~3.4 kb and ~6.4 kb fragments. This implied that the ~6.5 kb Sma I fragment was adjacent to the Sma I restriction site within the left arm and contained the left arm cloning junction. Not I cleavage of the ~6.5 kb Sma I fragment yielded a ~0.7 kb fragment and a ~5.8 kb fragment. The latter appeared as a doublet on the gel as it co-migrated with the ~5.7 kb Sma I fragment from the right arm of the vector. The ~2.15 kb and ~3.0 kb fragments were common to both the Sma I and Sma I/Not I digestions indicating that these Sma I fragments resulted from digestion within the insert. A summary of the Sma I/Not I digest fragments is shown in table 4.

The fragments produced by Eco RI and Sma I digestion are shown in table 4. Comparison of the Eco RI/Sma I digestion profile to the Eco RI and Sma I single digests

indicated that the ~10.3, 8.8 and 4.2 kb fragments produced by Eco RI digestion and the ~9.8, 6.5 and 3.0 kb Sma I fragments were absent from the double digestion profile. This indicated that these fragments had been cleaved in the Eco RI/Sma I double digestion. The ~10.3 kb Eco RI fragment has been proposed to contain the right arm which has an internal Sma I restriction site. From the Not I/Eco RI digestion, an Eco RI restriction site has been tentatively mapped ~1.2 kb from the end of the right arm. Together, this data suggested that the ~4.6 kb Eco RI/Sma I fragment was generated by Sma I cleavage of the ~10.3 kb Eco RI fragment ~3.4 kb from the cloning junction (Figure 11). If Eco RI cleaved the ~9.8 kb Sma I fragment once, a ~5.2 kb Eco RI/Sma I fragment would be expected. Because this fragment was not present within the double digestion profile, it was concluded that there was more than one Eco RI restriction site within this region of the clone. Furthermore, the ~8.8 kb Eco RI fragment did not lie directly adjacent to the right arm as this would have produced a ~5.2 kb fragment when cleaved with Eco RI and Sma I. It had been concluded from the Not I/Eco RI digest that an Eco RI restriction site must be ~1.0 kb or ~1.2 kb from the end of the left arm. This explained the absence of the ~6.5 kb Sma I fragment from the double digest. Eco RI cleaved this fragment, generating a ~1.7 kb fragment located between the Sma I site within the left arm and the Eco RI restriction site in the insert. A ~4.8 kb Eco RI/Sma I fragment would be predicted if Eco RI cleaved once within the ~6.5 kb fragment. The presence of this ~4.8 kb fragment in the digestion profile supported the proposal of one Eco RI site located within this fragment ~1.0 kb from the left arm cloning junction. This also implied that the ~8.8 kb Eco RI fragment lay adjacent to the left arm because the other Eco RI fragments were too small to produce a ~4.8 kb Eco RI/Sma I fragment. This result accounted for the Sma I cleavage of the ~8.8 kb Eco RI fragment in the double digestion (Figure 11). As the ~4.2 kb Eco RI fragment and not the ~2.0 kb Eco RI fragment was cleaved by Sma I, the ~4.2 kb fragment was positioned adjacent to the ~8.8 kb fragment. Therefore, based upon the ~6.2 kb Eco RI partial digestion product discussed earlier, the ~2.0 kb Eco RI fragment lay between the right arm Eco RI fragment and the ~4.2 kb fragment. A ~2.15 kb and a ~3.0 kb Sma I fragment could not be unaccounted for on the restriction map, although the ~3.0 kb Sma I fragment was cleaved by Eco RI. It was proposed from the position of the other Sma I restriction sites within λOHNZ1 that these Sma I fragments, ~2.15 kb and ~3.0 kb, were adjacent to each other within the clone. The ~3.0 kb fragment was likely to be located beside the ~9.8 kb Sma I fragment because this was the only possible site within the clone to explain the cleavage of this fragment by Eco RI. This positioned the ~2.15 kb Sma I fragment between the ~6.5 kb and ~3.0 kb Sma I fragments (Figure 11). The suggested positions of the Eco RI and Sma I restriction sites were in reasonable agreement with the observed fragments produced in the Eco RI/Sma I digestion; ~19.3, 5.7, 4.8, 4.6, 2.95, 2.15, 1.95, 1.7, 1.55 and 1.18 kb. The ~2.15 kb fragment was produced by Sma I digestion and the

~1.95 kb by Eco RI digestion alone. The origin of the ~19.3, 5.7 and 4.6 kb fragments had been established previously. Sma I digestion within the ~4.2 kb Eco RI fragment was likely to generate the ~2.95 kb fragment, although the current map would have predicted a ~3.15 kb fragment. The ~1.18 kb fragment was possibly the other digestion fragment produced by Sma I cleavage of the ~4.2 kb Eco RI fragment. The expected value for this fragment deduced from the restriction map was ~1.05 kb. In the lower region of the agarose gel, the fragments appeared to be broad and diffuse making the calculation of fragment sizes and number of fragments inprecise. This could have also been a source of error for the ~1.18 kb fragment. A discrepancy existed between an expected 1.95 kb and an observed 1.55 kb fragment generated by the Eco RI cleavage of the ~3.0 kb Sma I fragment. This possibly indicated an inaccuracy within the restriction map at this time.

Digestion with Sma I/Bam HI produced fragments of ~19.3, 8.5, 5.7, 2.2, 1.85, 1.65, 1.55, 1.25, 1.0 and ~0.62 kb. A low total molecular size (43.62 kb) for this digest implied that there were undetected fragments in this digestion or inaccurate size determination of the detected fragments. This may have been caused by the high level of RNA visible at the bottom of the gel and the broad diffuse appearance of the lower fragments. The ~1.65 kb Sma I/Bam HI band was broader and had a brighter intensity than a similar fragment in the adjacent Not I/Bam HI digestion profile. This may have indicated the presence of two or three ~1.65 kb fragments co-migrating during electrophoresis. This possibly accounted for the low total molecular size value obtained. More definite separation and visualisation of fragments within this region of the gel would be necessary to resolve this discrepancy. A detailed knowledge of the location of Bam HI restriction sites within the clone would also aid in determining whether the ~1.65 kb Sma I/Bam HI band represented multiple fragments. Fragments produced by specific Bam HI digestion could not be identified in the Sma I/Bam HI profile because a Bam HI single digest was not performed in this experiment. Consequently the ~1.85, 1.65, 1.55, 1.25, 1.0 and 0.62 kb fragments could not be placed within the restriction map. Comparison of the Not I/Bam HI and Eco RI/Bam HI digests (Table 4) implied that the ~2.25, 1.6, 1.1 and 0.62 kb fragments were produced by Bam HI cleavage, as these fragments were common to both digestions. The origin of the ~2.2 kb fragment in the Sma I/Bam HI digest could not be unequivocally assigned without knowledge of a Bam HI single digestion profile. The presence of a ~2.2 kb fragment in the Sma I single digestion possibly accounted for a fragment of this size in the Sma I/Bam HI digestion. The ~19.3 kb and ~5.7 kb fragments were produced by Sma I digestion as previously discussed. Because Bam HI does not cleave within the λFIX II vector (Figure 9), it was likely that the unique ~8.5 kb Sma I/Bam HI fragment was generated by a large Bam HI fragment (including the right arm of the vector) being cleaved by Sma I. This suggested

the presence of a ~14.2 kb Bam HI fragment (8.5+5.7 kb). Based upon the current restriction map (Figure 11), this fragment would be cleaved twice by Eco RI and once by Not I. The presence of this proposed ~14.2 kb Bam HI fragment was supported by the presence of ~5.0 kb and ~9.1 kb Not I/Bam HI fragments which would have resulted from Not I cleavage of such a fragment. The position of this putative Bam HI restriction site was also consistent with the cleavage of the ~4.2 kb Eco RI fragment by Bam HI and the apparent absence of Bam HI sites within the ~2.0 kb Eco RI fragment (Figures 10A & 11). The origin of the remaining ~2.80, 2.65 and 1.25 kb fragments in the Not I/Bam HI digestion could not be determined from the existing data.

Eco RI/Bam HI digestion of λOHNZ1 produced fragments with molecular sizes of >20, ~10.70, 2.70, 2.45, 2.25, 2.00, 1.73, 1.60, 1.10 and 0.62 kb. The exact size of the >20 kb fragment in this digest could not be determined although interpretation of the Eco RI digestion suggested that this fragment could not exceed ~21 kb in size (page 147). An approximate, ~21 kb value has been included in this digest profile (Table 4). Subsequent restriction analysis confirmed that this ~21 kb value was the correct length for this fragment (Figure 14). The ~10.70 kb fragment was generated by Eco RI digestion as it had the same mobility as a fragment within the Eco RI digestion profile which had earlier been proposed to be a ~10.3 kb fragment. A ~3.9 kb Eco RI/Bam HI fragment would have been produced if only the ~10.3 kb Eco RI restriction site was present in the proposed ~14.2 kb Bam HI fragment. However, the absence of the ~3.9 kb fragment agreed with the restriction map which predicted two Eco RI restriction sites within the ~14.2 kb Bam HI fragment (Figure 11). Accordingly, comparison of the Eco RI and Bam HI/Eco RI digestions implied that the ~2.0 kb fragment in the double digestion was produced by Eco RI digestion. The position of the other Eco RI/Bam HI fragments, ~2.7, 2.45, 2.25, 1.73, 1.60, 1.10 and 0.62 kb could not be assigned to the restriction map without additional data.

The Xba I and Xba I/Not I digestion profiles were identical because Xba I and Not I restriction sites are adjacent on the arms of λFIX II vector. This was also true for the Sst I and Sst I/Not I digestions. As a result of this, the 9.1 kb and 20 kb fragments present in the Xba I, Xba I/Not I and Sst I/Not I digestions represented the arms of the vector. Fragments of lower molecular sizes, ~6.6, 3.2, 2.6, and 2.2 kb for digestions containing Xba I and ~4.8, 2.8, 2.6 and 2.25 kb for Sst I/Not I, were produced by digestion within the insert. Double digestions involving Sst I and Xba I with other restriction enzymes would be required to establish the position of these fragments within λOHNZ1. The origin of a ~4.05 kb partial digestion product within the Sst I/Not I profile was not able to be determined. The sum of the fragments generated by Xba I,

Xba I/Not I and Sst I/Not I digests were lower than the other restriction digests (Table 4). This implied that fragments were unaccounted for in these profiles.

The interpretation of the data obtained from the digestion profiles (Figure 10A) is shown schematically in figure 11. Table 4 shows that the total size of λOHNZ1 varies between digestion profiles. The DNA fragments on the gel shown in figure 10A were transferred to GeneScreenPlus nylon membrane, as previously described in section 2.2.11. A cDNA probe comprising exon I sequences (described in section 3.2) was hybridised to the blot as described (Section 2.2.9).

Specific hybridisation signals were present in all λOHNZ1 lanes (Figure 10B, Table 5). A ~17.3 kb fragment hybridised in the Not I digestion providing further evidence that this fragment represented the insert. In both the Eco RI and Not I/Eco RI digestions, a ~8.8 kb fragment hybridised confirming that this fragment was produced by Eco RI digestion. A ~6.5 kb Sma I fragment hybridised to the exon I probe. This fragment was cleaved by both Eco RI and Not I as fragments of lower molecular sizes hybridised in the double digestions of these enzymes with Sma I. The Not I/Sma I fragment was ~5.8 kb and could be accounted for by one of the Sma I sites of the ~6.5 kb fragment lying ~0.7 kb within the left arm (Figure 9). Therefore, the fragments which hybridised to exon I lay adjacent to the left arm of the vector (Figure 12). The ~4.8 kb Sma I/Eco RI fragment was produced by Eco RI cleavage of the ~6.5 kb Sma I fragment. Since Not I/Eco RI digestion produced unique ~1.2 kb and ~0.98 kb fragments, the Eco RI site within the ~6.5 kb Sma I fragment must have been located at the end closest to the Not I to be consistent with both pieces of data. Using the Eco RI site deduced from the Sma I digest data, another Eco RI site was positioned 8.8 kb upstream. This suggested that Not I/Eco RI digestion at the left arm released the ~0.98 kb Not I/Eco RI fragment. This preliminary map accounted for both the 8.8 kb Eco RI and 6.5 kb Sma I fragments hybridising to the exon I probe. Additional information regarding the relative position of Sst I and Xba I to Eco RI and Sma I cleavage sites was required before the position of the 2.8 kb Sst I and ~6.6 kb Xba I and Xba I/Not I fragments could be assigned. As these fragments also hybridised to the exon I probe they should be located within the same region of the clone as the ~8.8 kb Eco RI and ~6.5 kb Sma I fragments. All digestions containing Bam HI gave a hybridisation signal correlating to a ~0.62 kb fragment. The position of this fragment relative to other known restriction fragments could not be established using the data presented thus far. Subsequent restriction analysis of a subcloned fragment has defined the location of the ~0.62 kb Bam HI fragment as being approximately 4.9 kb from the left arm in λOHNZ1 (Section 3.5). The hybridisation data is summarised in figure 12.

A 3.2 Analysis of figure 13 (Page 46)

Further restriction endonuclease digestions of λOHNZ1 were performed to clarify some of the anomalies identified within the previous gel discussion. The digestion profiles are shown in figure 13A.

Figure 10A indicated that Not I digestion released the insert intact from λOHNZ1. Therefore, only three fragments were expected from the Not I digest on the agarose gel in figure 13A. The Not I digestion profile however showed three dark bands at ~19.6, 17.0 and 10.2 kb and several faint, smaller, fragments. Both the ~19.6 kb and ~17 kb fragments were in reasonable agreement with the expected size of the left arm (~20 kb) of the vector and that deduced for the insert (17.3 kb) from the previous gel. The right arm should have had a molecular size of ~9.1 kb, indicating some degree of error within the estimated ~10.2 kb value (Table 6). If the 19.6 kb and 10.2 kb fragments were assumed to be ~20 kb and 9.1 kb respectively, a total molecular size of ~46.1 kb would have been obtained for this digestion. The faint fragments observed below the ~9.1 kb arm were possibly caused by contamination within either the λ preparation or the restriction digest, or through contamination of the digest samples with the 1 kb BRL DNA ladder. Consequently these fragments were ignored.

Eco RI produced a digestion profile containing fragments of ~21, 10.8, 8.8, 4.15 and 2.0 kb which have been discussed previously and mapped. The Not I/Eco RI digestion pattern was consistent with that discussed previously. Resolution of the ~9.1 kb and ~8.8 kb fragments has been demonstrated in figure 13A, confirming the presence of a doublet in this digest on the previous gel. It was probably that the faint fragments which were visible within the Not I/Eco RI digestion profile arose in a similar manner as those observed in the Not I digestion. These fragments were ignored.

Bam HI digestion of λOHNZ1 produced eight visible fragments; >20, ~14.5, 2.85, 2.75, 2.2, 1.58, 1.15 and 0.74 kb. The ~14.5 kb fragment, predicted in the previous gel discussion to be ~14.2 kb, confirmed that a large Bam HI fragment existed within the region of the right arm of the clone. This fragment also confirmed that the ~8.5 kb Sma I/Bam HI fragment was generated by a Sma I site within the right arm and a Bam HI restriction site ~5.0 kb from the cloning junction at the right arm. Comparison of the Bam HI and Not I/Bam HI digestions (Figure 13A) established that both the ~14.5 kb and >20 kb Bam HI fragments were cleaved by Not I and thus must have been produced by the unique Not I/Bam HI fragments; ~20, 9.5, 5.0 and 1.28 kb. The size of the Bam HI fragment (14.5 kb) suggested that this fragment contained the right arm of the vector. This was confirmed by the presence of the ~9.5 kb fragment corresponding to the right arm in the Bam HI/Not I digest. To account for the ambiguous mobility of

the right arm fragment, a value of ~9.1 kb was used to calculate the molecular size total for this digestion. The ~5.0 kb fragment accounted for the remainder of the ~14.2 - 14.5 kb Bam HI fragment. The ~1.28 kb fragment was generated by Not I cleavage at the left arm. From this data, it was predicted that a ~21.28 kb Bam HI fragment could be cleaved by Not I and this would release the observed ~1.28 kb Not I/Bam HI fragment. This placed a Bam HI restriction site ~1.28 kb from the left arm cloning junction. The fragments common to both the Not I/Bam HI and Bam HI digests (~2.85, 2.75, 2.2, 1.58, 1.15 and 0.74 kb) resulted from Bam HI digestion within the insert since the λFIX II vector was not cleaved by Bam HI.

Double digests with Eco RI and Bam HI produced a profile comparable to that previously observed; ~21, 10.8, 2.75, 2.45, 2.2, 2.0, 1.73, 1.58 and 1.15 kb. An additional ~0.78 kb fragment which was not observed in the gel shown in figure 13A, was visible in this digestion. It was assumed that this fragment was the same as the ~0.74 kb Bam HI and Not I/Bam HI fragment, possessing a different size due to an artefact in electrophoresis. The Bam HI restriction site located ~1.28 kb from the left arm confirmed that the left arm fragment in the Eco RI/Bam HI digest was ~21 kb in size and was produced by Eco RI cleavage. From the position of the Eco RI and Bam HI restriction sites at the left arm, a ~0.28 kb Eco RI/Bam HI fragment would have also been expected to be produced by Eco RI/Bam HI digestion. This ~0.28 kb fragment was not visible in figure 13A as it had run off the gel. The ~8.8 kb and ~4.15 kb fragments produced by Eco RI cleavage and the ~14.5 kb and ~2.85 kb fragments visible in the Bam HI digest were absent from the Eco RI/Bam HI double digestion. The Bam HI restriction site which generated the ~14.5 kb fragment was located within the ~4.15 kb Eco RI fragment. Two Eco RI restriction sites were located within the ~14.5 kb Bam HI fragment (Figure 14). Cleavage at these sites released the ~10.8 kb and ~2.0 kb Eco RI fragments. The unique Eco RI/Bam HI fragment generated by this digestion was predicted to be ~1.9 kb and was possibly represented by the ~1.73 kb fragment. As the precise location of all Bam HI restriction sites within λOHNZ1 had not been established, it could not be determined whether the ~4.2 kb Eco RI fragment contained more than one Bam HI site. The presence of a ~2.45 kb Eco RI/Bam HI fragment implied that a single Bam HI site may have been located within this region. This fragment had a similar size to a 2.3 kb fragment predicted to result from such an arrangement of restriction sites. The ~2.85 kb Bam HI fragment was cleaved by Eco RI and possibly generated the ~2.45 kb Eco RI/Bam HI fragment. For this to occur, the ~2.85 kb Bam HI fragment must have been adjacent to the ~14.5 kb Bam HI fragment. When the map was drawn in this manner (Figure 14), a ~0.55 kb Eco RI/Bam HI fragment was expected from the Eco RI cleavage of the ~2.85 kb Bam HI fragment. This fragment was not observed, indicating

an error in the positioning of the restriction sites or in the size determination of the digestion fragments. It was established previously that the left arm Bam HI fragment was ~21.28 kb. The ~2.85 kb Bam HI fragment positioned next to the ~14.5 kb Bam HI fragment contained the junction of the ~8.8 kb and ~4.15 kb Eco RI fragments. Together the ~21.28 kb and ~2.85 kb Bam HI fragments accounted for two Bam HI cleavage sites within the ~8.8 kb Eco RI fragment. Additional Bam HI restriction sites must have been contained within the ~8.8 kb fragment to explain the size of the other Eco RI/Bam HI digestion products. These Bam HI restriction sites were likely to produce the remaining ~2.75, 2.2, 1.58, 1.15 and 0.78 kb Bam HI fragments.

The fragments produced by the Sma I/Eco RI digestion were comparable to previous results (Table 6). The ~1.18 kb fragment observed in an earlier digest (Figure 10A) was re-assigned a fragment size of ~1.08 kb which was in close agreement with the size of ~1.1 kb suggested previously.

The ~0.7 kb fragment produced by the Not I cleavage of the ~6.5 kb Sma I fragment was clearly visible within the Sma I/Not I digestion (Figure 13A). The other Sma I/Not I fragments were mapped in accordance with the previous data.

Twelve Sma I/Bam HI fragments (~19.3, 8.6, 5.7, 2.2, 1.95, 1.65, 1.58, 1.52, 1.25, 1.2, 1.0 and 0.68 kb) were visible on the agarose gel in figure 13A compared to nine fragments previously observed (Table 4). The position of the ~19.3, 8.6 kb and 5.7 kb fragments had been assigned earlier (page 148). Because a Bam HI restriction site was ~1.28 kb from the left arm, the ~1.95 kb Sma I/Bam HI fragment was generated by cleavage at this Bam HI site and the Sma I restriction site within the left arm (Figure 14). Since the ~14.3 - 14.5 kb Bam HI fragment had also been positioned, it could be postulated that the ~1.25 kb Sma I/Bam HI fragment was produced by Bam HI cleavage the ~9.8 kb Sma I fragment observed on the previous gel (Figure 10A). The positioning of the ~2.85 kb Bam HI fragment was supported by the presence of a ~1.65 kb Sma I/Bam HI fragment. This was in accordance with both the ~2.85 kb Bam HI and ~3.0 kb Sma I fragments being cleaved within the Sma I/Bam HI digest. Comparison of the Sma I digestion products (Table 4) and the Bam HI digest fragments (Table 6) showed that a ~2.2 kb fragment was present in both digestions. A ~2.2 kb fragment with the same band intensity within the Sma I/Bam HI digest implied that at least one of the ~2.2 kb fragments present in the single digestions had been cleaved within the double digestion. As the Bam HI restriction sites were not characterised within λOHNZ1, the origin of the ~2.2 kb fragment within the double digestion could not be determined. Again, the ~0.68 kb fragment was thought to be the same fragment as the ~0.78 - 0.72 kb fragment visible

in the other Bam HI digestions. The ~1.52, 1.2 and ~1.0 kb fragments produced by Sma I/Bam HI digestion could not be positioned on the restriction map.

The Sst I and Sst I/Not I digestions gave identical digestion profiles (~20, 9.8, 4.9, 2.9, 2.65, 2.2, 0.86 and 0.74 kb) since there was a site for Sst I adjacent to the Not I site at the cloning junction. This profile was consistent with that previously observed with the addition of the ~0.86 kb and ~0.74 kb fragments. These fragments were not detected earlier due to either the presence of RNA or insufficient DNA to visualise fragments of this size. The ~9.8 kb fragment represented the 9.1 kb right arm. A lower salt concentration in the Sst I digest caused the fragments within this profile to migrate with a slightly higher mobility than the Sst I/Not I fragments (Figure 13A). The intensity of the ~2.65 kb fragment relative to the ~2.90 kb and ~2.2 kb fragments, indicated that a doublet of this size was present in these digestions.

It had been established previously that Xba I digestion resulted in fragments with molecular sizes of ~20, 9.1, 6.6, 3.2, 2.6 and ~2.25 kb (Table 4). The Xba I and Xba I/Not I digestion profiles shown in figure 13A contained a doublet at ~2.65 kb. Several partial digestion products (Table 6) were detected as faint fragments within the Xba I and Xba I/Not I digests shown in figure 13A. The 3.0 kb difference between the 17 kb and 14 kb partial bands suggested that complete digestion may lead to the release of the ~3.2 kb Xba I fragment. The same situation may have applied to the ~9.8 kb partial and the ~6.6 kb complete digestion fragments. Likewise, the 5 kb and 3.6 kb partial fragments may have produced one of the 2.6 - 2.65 kb Xba I fragments. The difference in sizes between the 5.4 kb and 5.0 kb bands, 3.6 kb and 3.0 kb bands and the 3.2 kb complete fragment and the 2.85 kb partial band suggested the presence of a 0.2-0.4 kb Xba I fragment which was too small to be visualised.

Fragments were transferred to GeneScreen nylon membrane and hybridised to a radiolabelled probe comprising of exon II sequences (Section 3.1). Table 7 summarises the fragments which hybridised to the probe (Figure 13B). All Bam HI digestions gave a hybridisation signal corresponding to a ~1.58 kb fragment indicating that exon II was contained within a discrete Bam HI fragment. Three fragments hybridised to the probe in the Xba I digestion. The top two fragments (~17.0 kb and ~9.8 kb) were partial digestion products and produced a weaker hybridisation signal than the lower ~6.6 kb fragment. The presence of these partial digestion products (~17.0 kb and 9.8 kb) suggested that the ~3.2 kb Xba I fragment lay adjacent to the ~6.6 kb fragment. The position of the ~6.6 kb Xba I, 4.9 kb Sst I and 1.58 kb Bam HI fragments could not be determined from this data. Since the ~8.8 kb Eco RI, ~5.8 kb Sma I/Not I, ~4.8 kb Sma I/Eco RI and ~6.6 kb Xba I fragments hybridised to both exon I and exon II probes, it

could be inferred that these fragments contained both of these regions of the gene (Figures 12 & 15). It was concluded that both Sst I and Bam HI cleaved between exon I and exon II based upon fragments of different sizes hybridising to the exon I and exon II probes.

A 3.3 Analysis of figure 16 (Page 50)

Previously, restriction fragments had been oriented by comparison of single digests to double digestions with Not I and the enzyme in question. Fragments cleaved by Not I were identified and mapped to the appropriate region of the clone. This approach was inappropriate for Sst I and Xba I as the restriction sites for these enzymes were located adjacent to the Not I site within the vector and thus produced identical digestion profiles for both single digests and double digests with Not I. To enable the Sst I and Xba I restriction sites to be mapped within λOHNZ1, the digest fragments were oriented relative to other defined restriction sites, such as Eco RI and Sma I. Double digestions involving Eco RI, Sma I, Sst I and Xba I were performed and the resulting fragments separated by agarose gel electrophoresis (Figure 16A).

The Eco RI, Sst I and Sma I single digestion profiles (Table 8) were in accordance with previous results. The Eco RI and Sma I fragments were mapped as determined previously (Figure 11). Some variation in molecular sizes of digest fragments was apparent between the values calculated from the current data and those deduced from earlier experiments. In most instances the current gel fragment sizes were mapped according to the overall cleavage patterns and prior gel interpretations. The complex partial digestion products in the Xba I digestion profile (Table 8) precluded any correlation between these fragments and the complete digestion fragments. The origin of two partial digestion fragments of ~12.8 kb and ~10.8 kb in the Sma I digestion were similarly unable to be assigned. The Sst I digest produced fragments with a total size of 44.25 kb (Table 8) which was less than that observed in other restriction digestions. This may have indicated that some undetected fragments were present within this digestion. A ~0.76 kb and ~0.59 kb fragment present within the Sst I/Eco RI digestion profile may have originated from Sst I digestion as there were faint fragments of this molecular size in the Sst I digest. Inclusion of these fragments within the Sst I profile gave a total size of ~45.70 kb which was still lower than the other digestions. This inconsistency was likely to result in inaccuracies in the positioning of the detected Sst I fragments which may been apparent as discrepancies between observed and predicted fragment sizes within double digestions. Comparison of the Sst I, Xba I and Sst I/Xba I digests showed the ~2.65 kb band had a brighter intensity in the single digestions than

the Sst I/Xba I digest. This indicated that at least two fragments were co-migrating at ~2.65 kb in the each of the single digestions.

As Sst I, Xba I and Not I restriction sites were adjacent in the vector, the fragments of ~19.3, 3.4 and 0.7 kb in the Sst I/Sma I and Xba I/Sma I double digests were probably generated in a similar manner to fragments of these sizes produced in the Sma I/Not I digest (Figure 13A). The ~5.7 kb fragment was a product of Sma I digestion within the right arm and accounted for the absence of the ~9.1 kb arm in these digestions. The ~20 kb and 9.1 kb fragments in the Sst I/Eco RI, Xba I/Eco RI and Sst I/Xba I digestions represented the arms of the vector and resulted from Sst I and/or Xba I cleavage at the cloning junctions. The other fragments in these digestions were derived from restriction sites within the insert.

Comparison of the Sma I and Sma I/Xba I digestion profiles showed that a doublet was present within the double digestion at ~5.7 kb (Figure 16A). This was also observed in the previous Sma I/Not I digestion and lead to the ~6.5 kb Sma I fragment being mapped across the left arm cloning junction. Earlier hybridisation experiments showed that the ~6.5 kb Sma I and ~6.6 kb Xba I (a 6.4 kb fragment on the current gel) fragments were located within the same region of λOHNZ1. Thus, the ~5.7 kb doublet must have resulted from the ~6.5 kb Sma I fragment being cleaved once by Xba I. Furthermore, the Xba I restriction site was located at the cloning junction. A ~0.7 kb Sma I/Xba I fragment was also released by this digestion. This suggested that the ~6.4 kb Xba I fragment was located beside the left arm. Positioned in this manner, the ~6.4 kb Xba I fragment would be cleaved by the Sma I restriction site within the insert, generating a ~0.6 kb Xba I/Sma I fragment. Supporting these findings was the absence of the ~6.4 kb Xba I and ~6.5 kb Sma I fragments from the double digestion profile.

With the location of the ~6.4 kb Xba I fragment within λOHNZ1 known and from the partial Xba I fragments which hybridised to the exon II probe, the ~3.2 kb Xba I fragment could be mapped adjacent to the ~6.4 kb Xba I fragment. Therefore a Xba I restriction site lay within both the ~2.1 kb and ~3.0 kb Sma I fragments. Cleavage of the ~2.1 kb Sma I fragment by Xba I produced fragments of ~0.6 kb and ~1.5 kb, which confirmed that predicted from the restriction map (Figure 17). The presence of the ~1.7 kb Sma I/Xba I fragment was in agreement with the predicted size for the digestion product formed by Sma I cleavage of the ~3.2 kb Xba I fragment. The remainder of the ~3.0 kb Sma I fragment was likely to be represented by a ~1.2 kb Sma I/Xba I fragment which could be seen on the gel (Figure 16A). Since the ~2.2 kb Xba I fragment was also cleaved by Sma I, it was positioned next to the ~3.2 kb Xba I fragment (Figure 17). Sma I digestion of this fragment was expected to yield a ~1.2 kb fragment and a ~1.0 kb

fragment. Neither of the two ~2.65 kb Xba I fragments appeared to be cleaved by Sma I which was consistent with these fragments lying between the Sma I site and the right arm (Figure 17).

The size of several Eco RI/Xba I digest products were predicted using the location of the ~6.4 kb and ~3.2 kb Xba I fragments within the clone. Eco RI cleavage of the ~6.4 kb Xba I fragment produced ~0.96 kb and ~5.4 kb fragments. According to the position of the Eco RI and Xba I restriction sites within λOHNZ1, the ~3.2 kb Xba I fragment should not have been cleaved by Eco RI. This fragment however appeared to have a lower molecular size in the Xba I/Eco RI profile (3.05 kb) than the corresponding fragment in the Xba I single digestion. Generally an increase in the mobility of a fragment was an indicator that the fragment had been cleaved. The lower molecular size of the ~3.2 kb fragment may have accounted in part for the reduced total molecular size in this digestion. Comparison of the ~20 kb left arm fragment in the Sst I and Xba I digests, indicated that this ~20 kb fragment had a different mobility in the Eco RI/Xba I profile. This anomaly was possibly caused by lower concentrations of DNA within this digest as the salt concentrations were identical in the Eco RI/Xba I and Xba I digestions. The Eco RI/Xba I fragments appeared as sharp bands compared to the smeared fragments visible in the other digestions (Figure 16A). The presence of sharp bands indicated that there was a lower concentration of DNA within this digest than the other digestions, while the smearing of fragments implied that there were higher levels of DNA. The difference in mobility of the Eco RI/Xba I fragments may have accounted for the apparent cleavage of the ~3.2 kb Xba I fragment by Eco RI. To compensate for the mobility difference of the ~20 kb left arm, a value of 20 kb was tabulated (Table 8) and used to calculate the total size of fragments in this digestion. The restriction map constructed thus far showed an Eco RI restriction site within the ~2.2 kb Xba I fragment and this cleavage may have explained the presence of a ~1.85 kb Xba I/Eco RI fragment (Figure 17). An ~0.35 kb Eco RI/Xba I fragment would also have been produced by this digestion and therefore a fragment of this size has been included in this digestion profile (Table 8). However, there was insufficient DNA present in this digestion to observe a fragment of this size. The ~2.15 kb fragment was possibly formed by Xba I cleaving the ~4.0 kb Eco RI fragment. As a consequence of the concentration artefact in this digest, the other fragments (1.28 kb and 1.18 kb) could not be assigned to the restriction map. It could be concluded however, that these fragments lay near the right arm and thus explained the absence of the ~2.0 kb Eco RI and both of the ~2.65 kb Xba I fragments from the double digestion profile (Figure 16A). It was possible that either the ~1.28 kb or the ~1.18 kb Xba I/Eco RI fragment were produced by Xba I cleavage at the right arm cloning site and

Eco RI at the adjacent site. The origin of the Xba I/Eco RI partial digestion fragments (~15.3, 11.7, 10.5, 8.6 kb) were unknown.

Earlier hybridisation experiments had shown that exon II lay within a ~4.9 kb Sst I fragment and exon I was in a ~2.85 kb Sst I fragment, suggesting that Sst I cleaved within intron I. Accordingly, the ~8.8 kb Eco RI, ~6.5 kb Sma I and ~6.4 kb Xba I fragments which hybridised to both exon I and exon II, contained Sst I restriction sites. The ~4.9 kb Sst I fragment was present in the Sst I/Sma I and Sst I/Xba I digestions but was cleaved in the Sst I/Eco RI digest, implying the ~8.8 kb Eco RI and ~4.9 kb Sst I fragments overlapped (Figure 16A). As the position of Sma I, Xba I and Eco RI restriction sites had been determined in the region of λOHNZ1 which contained exon I and exon II, it was possible that the ~4.9 kb Sst I was next to the left arm. The presence of a ~3.85 kb Sst I/Eco RI fragment suggested that Eco RI cleaved ~1.05 kb from the end of the ~4.9 kb fragment. This was supported by the presence of a ~1.1 kb fragment within the Sst I/Eco RI digestion profile and was in agreement with the Eco RI restriction site which was positioned ~1.0 kb from the left arm. Extrapolating from the data, the ~2.85 kb Sst I fragment which was digested by Sma I and Xba I, would have been adjacent to the ~4.9 kb fragment to be consistent with the earlier hybridisation data (Figure 12). The presence of the ~2.85 kb fragment in the Sst I/Eco RI digestion indicated that this fragment was contained within the ~8.8 kb fragment. The position of the other Sst I fragments (~2.65, 2.65, 2.20, 0.76, 0.59 kb) within λOHNZ1 could not be established without additional information. The source of the ~11.5 kb partial fragment and the ~1.55 kb and ~1.3 kb Sst I/Eco RI fragments was unknown. Sst I digestion of the ~10.8 kb Eco RI fragment, (thought to be a ~10.3 kb fragment migrating anomalously) may have occured in a manner similar to the previously described Not I cleavage of this fragment. If this was so, cleavage of this fragment may have accounted for the presence of the ~1.3 kb Sst I/Eco RI fragment. Overall, Sst I appeared to cleave all Eco RI fragments with the possible exception of the ~2.2 kb fragment. The order of the ~4.9 kb and 2.85 kb Sst I fragments in λOHNZ1, suggested that the insert had been cloned into the λFIX II vector in a 3' to 5' orientation, relative to the left and right arms. That is, exon II was located nearest the left arm and exon I towards the right arm.

With the ~4.9 kb and ~2.85 kb Sst I fragments mapped (Figure 17), it was possible to predict the size of some of the double digestion fragments produced by Sst I and other restriction enzymes. The presence of these fragments supported the positioning of the Sst I fragments in the clone.

It was likely that two Sst I restriction sites were located within the ~6.5 kb Sma I and ~6.4 kb Xba I fragments. The restriction map indicated that Sst I digestion of the ~6.5 kb Sma I fragment would produce fragments of ~0.7, ~4.9 and ~0.87 kb while Sst

I cleavage of the ~6.4 kb Xba I fragment would yield fragments of ~4.9 kb and ~1.5 kb. All of these fragments were visible in the respective double digestion profiles (Figure 16A). The observed ~2.0 kb Sst I/Sma I and ~1.25 kb Xba I/Sst I fragments were comparable to the predicted fragment sizes for the other cleavage products of the ~2.85 kb Sst I fragment. Inclusion of the ~0.76 kb Sst I fragment suggested a doublet within the Sma I/Sst I digest at ~0.7 kb. For this reason, both of these fragments were included in the total digestion profile (Table 8). However, it could not be established whether both of the ~0.76 kb and ~0.59 kb Sst I fragments were present within the Sst I/Sma I digest. Also within the Sma I/Sst I profile was a ~2.25 kb doublet and possibly one 2.65 kb fragment. Until the precise location of all Sst I restriction sites are known, the origin of the ~2.25 kb doublet and the exact number of 2.65 kb fragments present within this digestion cannot be established. Based upon the total molecular size of this digestion, it was proposed that a single 2.65 kb fragment was present within the Sst I/Sma I digest. Comparison of the Sst I/Sma I and Sma I digests showed that the ~3.0 kb and ~9.8 kb Sma I fragments were cleaved by Sst I (Figure 16A). A ~0.75 kb fragment visible in the Sst I/Xba I digest may have been either the ~0.76 kb or ~0.59 kb Sst I fragment. Better resolution and detection of fragments within this region of the gel would clarify this assignment. The origin of the ~2.2 kb fragment in the Sst I/Xba I profile could not be established without additional information as a fragment of this size was present in both single digestions. The remaining fragments in the Sst I/Xba I digestion (~2.65 kb and ~1.9 kb) were not informative.

The digestion fragments were transferred to a nylon membrane and hybridised to a probe representing exon I sequences. The resulting autoradiograph is shown in figure 16B and are summarised in table 9. The presence of the ~2.85 kb fragment in the Sst I/Eco RI hybridisation profile indicated that this fragment was produced by Sst I digestion within the ~8.8 kb Eco RI fragment. Fragments of ~0.87 kb and ~1.56 kb respectively, hybridised in the Sma I/Sst I and Sst I/Xba I digestions confirming the position of the Sma I and Xba I restriction sites in the ~2.85 kb Sst I fragment. This specified that exon I was contained within a ~0.87 kb Sst I/Sma I fragment. Xba I digestion produced a single hybridisation signal correlating to the ~6.4 kb fragment. This fragment was cleaved by Sma I releasing the ~5.8 kb Xba I/Sma I hybridisation fragment which confirmed that one Xba I restriction site was located within the ~6.5 kb Sma I fragment. The position of the Eco RI restriction site in the ~6.4 kb Xba I fragment explained the ~5.4 kb hybridisation fragment observed in the Xba I/Eco RI digest. A summary of the fragments which contained exon I is shown in figure 18.

3.4 Analysis of figure 19 (Page 54)

To further characterise the restriction map of λOHNZ1, digestions involving the estriction enzymes Kpn I, Xho I and Hind III were performed in conjunction with estriction endonucleases for which the restriction sites within λOHNZ1 had been lefined. As neither Xho I nor Hind III cleaved the arms of the λFIX II vector (Figure 9), fragments produced by digestion with these enzymes should have resulted from cleavage within the insert. Two Kpn I sites were located in the left arm at ~17.05 kb and ~18.56 kb (Figure 9). This explained the different mobility of the left arm in the Kpn I digests compared with the other digestion profiles (Figure 19). The expected ~1.51 kb fragment produced by Kpn I cleavage within the left arm, was visible as either the ~1.4 kb or ~1.6 kb fragments present in all Kpn I digestions. Since no other Kpn I restriction sites were known to be contained in the vector, it was likely that the other fragment, either 1.6 kb or 1.4 kb, was generated by Kpn I digestion within the insert.

Like most of the digestions separated on the current gel, the top fragment in the Kpn I digestion was uncut DNA and was excluded from the digestion (Table 10). Accordingly, Kpn I digestion yielded four fragments; ~17.05, 8.8, 1.6 and 1.4 kb. The total size of these fragments was 28.25 kb, highlighting a difference of ~17.1 kb between this digest and other digestions (Table 10). Because a ~17.05 kb fragment had been detected within the Kpn I digestion, it was likely that this fragment was co-migrating with another ~17 kb fragment. In earlier digests co-migrating fragments were apparent because of an anomalous band intensity. In the Kpn I digests this was not obvious since the 17 kb fragment ran at the top of the digest, however this was the most likely explanation for the "missing" 17 kb. A ~1.44 kb Kpn I/Not I fragment was predicted for the Not I cleavage at the cloning junction and Kpn I digestion within the left arm. Consequently, the ~1.4 kb fragment had a darker band intensity in this digest, indicating a doublet. The presence of a ~7.4 kb fragment and a ~1.44 kb fragment, and the absence of the ~8.8 kb Kpn I fragment from the Not I/Kpn I digest, implied that Not I cleaved the ~8.8 kb fragment, releasing these fragments. Thus, the ~8.8 kb Kpn I fragment contained the left arm cloning junction and lay adjacent to the predicted ~1.51 kb Kpn I fragment (Figure 20). The production of the ~9.1 kb and ~8.0 kb Kpn I/Not I fragments supported the co-migration of two ~17 kb fragments in the Kpn I single digestion. One of the ~17 kb Kpn I fragments released a ~9.1 kb fragment when cleaved by Not I, which suggested that this ~17.1 kb fragment contained the entire right arm and ~8.0 kb of the insert. Therefore, a Kpn I restriction site was located in the insert ~8.0 kb from the end of the right arm. Positioned in this manner, the Kpn I digestion had a ~1.9 kb gap between the ~8.8 kb and ~17.1 kb fragments (Figure 20). This suggested that the ~1.6 kb fragment lay within this area. A ~0.5 kb fragment was expected if the ~1.4 kb Kpn I fragment lay in this region of the clone. As there was sufficient DNA within each digest

profile to detect a ~0.5 kb fragment, the absence of this fragment suggested that the ~1.6 kb Kpn I fragment was located in the centre of the clone and the ~1.4 kb fragment represented the 1.51 kb fragment which was generated by cleavage within the left arm. A ~0.30 kb fragment may have also existed to account for the remainder of the ~1.9 kb partial fragment. As this ~0.30 kb fragment was generated by Kpn I digestion, it was included in the Kpn I profile (Table 10). The possible existence of this fragment in the Kpn I digest prevented the exact positioning of the ~1.6 kb fragment.

Non-specific degradation of the DNA in the Kpn I/Hind III digest appeared as a smear in this digestion profile (lane four, Figure 19). The unique ~10.2 kb and ~7 kb fragments visible in the Hind III/Kpn I digest provided further evidence supporting the existence of a doublet at ~17.1 kb in the Kpn I digest. As the position of each 17 kb fragment had already been established, a Hind III restriction site must have been located ~1.2 kb from the right arm. Hind III digestion yielded two fragments, one of ~10.2 kb and one greater than 20 kb, which were consistent with the presence of one Hind III restriction site in the clone. Assuming that the clone had a total size of ~46.4 kb, it was likely that the largest Hind III fragment was ~36.2 kb. As the Hind III/Eco RI digestion profile was virtually identical to the Eco RI single digestion, the Hind III restriction site must have been sufficiently close to an Eco RI restriction site so that the Eco RI digestion pattern was not altered. The earlier Not I/Eco RI digestion data had positioned an Eco RI restriction site ~1.2 kb from the right arm. The single Hind III site was probably close to this Eco RI site (Figure 20).

The Eco RI digestion contained a ~0.24 kb fragment which had not previously been detected. It was possible that this fragment was responsible for some of the discrepancies observed in the earlier double digestion profiles. There were a number of partial digestion products, fragments of ~7.4, 5.8, 5.5, 3.4 and 2.28 kb, which were present within the Eco RI digestion. These fragments were ignored. To characterise the position of the ~0.3-0.24 kb Eco RI fragment in λOHNZ1, the 5' most region of the insert which was most likely to contain this fragment would need to be subcloned and subjected to additional restriction mapping. Since this fragment did not appear to be located within the proximal promoter region, the exact position of this fragment was not further investigated.

In addition to the ~1.63, 1.4 and 17.05 kb Kpn I fragments and the ~1.95, 0.24, 4.2 and 10.3 kb Eco RI fragments, double digestion with Kpn I and Eco RI produced fragments of ~2.5, 6.4 and 0.48 kb. The ~2.5 kb Kpn I/Eco RI fragment lay between the Kpn I site at ~18.56 kb and the Eco RI site ~1.0 kb from the left arm (Figure 20). The ~6.4 kb Kpn I/Eco RI fragment was adjacent to the ~2.5 kb fragment, while the

remainder of the 8.8 kb Eco RI fragment was represented by the ~1.6 kb Kpn I fragment, the putative ~0.30 kb Kpn I fragment and the ~0.48 kb Kpn I/Eco RI fragment.

A ~4.9 kb fragment was released when λOHNZ1 was digested with Xho I (Figure 19). Because Xho I did not cleave the λFIX II vector, the presence of the ~4.9 kb fragment indicated that there were two Xho I restriction sites within the insert. Since digestion at two sites within a linear piece of DNA produces three fragments, it was concluded that the upper most band in the Xho I digest contained two fragments. There was insufficient data in this Xho I single digestion to establish the precise location of the Xho I restriction sites within the clone.

The presence of the 4.9 kb Xho I fragment and cleavage of the ~8.8 kb Eco RI fragment in the Eco RI/Xho I double digestion indicated that both of the Xho I restriction sites were contained within the 8.8 kb Eco RI fragment. The presence of the ~21, 10.3, 4.2 and 2.0 kb Eco RI fragments indicated that no Xho I restriction sites were contained within these fragments. As expected, the ~8.8 kb Eco RI fragment was cleaved by Xho I to produce three fragments (4.9, 3.1, 0.81 kb). The exact order of these fragments could not be determined from this digest.

Xba I digestion produced a pattern similar to that shown by earlier results (Table 10). It was suggested earlier that a ~0.2 kb Xba I fragment may exist. The presence of a faint fragment ~0.33 kb in this Xba I digestion supported this proposal, however the precise location of this fragment in λOHNZ1 could not be established.

The ~6.4 kb Xba I fragment was cleaved by Eco RI releasing a ~5.4 kb and a ~0.9 kb fragment. The agarose gel shown in figure 19 established that the 3.2 kb Xba I fragment was not cleaved by Eco RI, clarifying an earlier ambiguity. Both 2.6 kb Xba I fragments and the ~4.0 kb and ~2.0 kb Eco RI fragments were cleaved in the double digestion, giving rise to the ~1.83, 1.22, 1.15 and 0.66 kb Xba I/Eco RI fragments. Although there was sufficient DNA within this digestion to detect fragments of lower molecular sizes, the precise size and number of fragments generated in this digestion was not able to be established. This resulted in discrepancies in the restriction map. Since these fragments were not located in the region of primary interest, the additional experiments necessary to establish the precise location of these fragments were not carried out.

Comparison of the Xba I and Xho I/Xba I digestions showed that both the ~3.2 kb and ~6.4 kb Xba I fragments were cleaved by Xho I (Figure 19). This implied that the ~4.9 kb Xho I fragment contained an Xba I restriction site. As Xho I cleaved only in

the ~6.4 kb and ~3.2 kb Xba I fragments, the other fragments in the Xba I/Xho I digestion were generated by Xba I digestion and had identical mobilities to the corresponding fragments in the Xba I single digestion.

The position of the Xho I restriction sites was established by interpretation of both the Eco RI/Xho I and Xba I/Xho I digests. To obtain an ~0.81 kb Eco RI/Xho I fragment and an ~1.8 kb Xba I/Xho I fragment, a Xho I restriction site must have been located ~1.8 kb from the left arm. Accordingly, this suggested that the ~3.1 kb Eco RI/Xho I fragment was produced by cleavage at the 5' most end of the ~8.8 kb Eco RI fragment. The ~2.95 kb fragment present in the Xba I/Xho I profile resulted from Xho I cleavage ~0.25 kb from the end of the ~3.2 kb Xba I fragment. The ~4.65 kb fragment represented the remainder of the ~6.4 kb Xba I fragment together with most of the ~4.9 kb Xho I fragment (Figure 20). The sizes of the other Xho I fragments were deduced from the positions of the Xho I restriction sites mapped within λOHNZ1 (Table 10).

The size of the Xba I/Kpn I digest fragments were in agreement with those predicted from the positioning of the individual restriction sites. The ~3.2 kb Xba I fragment was cleaved, releasing a ~1.65 kb Kpn I fragment and ~0.81 kb and ~0.37 kb Xba I/Kpn I fragments (Figure 20).

The fragments were transferred to a nylon membrane and hybridised to an exon I radiolabelled probe to identify fragments which contained sequences that were complementary to this probe (Figure 21A). The membrane was washed to remove the bound probe and re-hybridised to an exon II radiolabelled probe. The resulting autoradiograph is shown in figure 21B. The fragments which hybridised to each probe is shown in table 11.

A high molecular size fragment (estimated to be ~21.8 kb) hybridised in the Xho I single digestion while a ~1.8 kb Xba I/Xho I fragment and a ~0.8 kb Xho I/Eco RI fragment also hybridised. Since Xho I fragments of different molecular sizes hybridise to the exon I and exon II probes, it was concluded that Xho I cleavesd between exon I and exon II. These results are summarised in figures 22 and 23.

Collectively, the exon I and exon II hybridisation patterns provided confirmation of the position of the digest fragments within the map. These results confirmed the earlier proposal that the insert was positioned in a 3' to 5' orientation relative to the left and right arms. Exon II was localised within a ~0.81 kb Xho I/Eco RI fragment which suggested that the ~1.58 kb Bam HI fragment which had earlier hybridised to exon II, was adjacent to the Bam HI restriction site near the left arm.

A 3.5 Analysis of figure 24 (Page 60)

The restriction endonuclease digestion profiles shown in figure 24 provided supporting data for the location of some additional restriction sites within λOHNZ1. The amount of DNA in each lane was such that the higher molecular size fragments appeared as broad overloaded bands while the ~3.0-0.5 kb fragments were identified clearly and sized accurately. Fragments less than 0.5 kb which had a diffuse appearance could not be sized accurately and consequently were ignored. However, previously observed fragments which were less than 0.5 kb in size were included in the size profiles of the appropriate digests. The Eco RI/Xho I, Eco RI, Eco RI/Kpn I and Xho I digestions all gave cleavage patterns comparable to those observed on earlier agarose gels (Table 12).

Eco RI cleavage of the ~4.9 kb Sst I fragment had earlier been predicted to release the ~3.9 kb and ~1.0 kb Eco RI/Sst I fragments. The ~4.0 kb fragment in the current Sst I/Eco RI profile presumably accounted for the predicted ~3.9 kb fragment, however the ~1.0 kb fragment was absent. In the Xba I/Eco RI digest on the previous gel (Figure 19), a ~0.9 kb Xba I/Eco RI fragment was found to correspond to the same region of the clone as the proposed ~1.0 kb Eco RI/Sst I fragment. Therefore, it was possible that the ~0.86 kb band in the Sst I/Eco RI digest, which had a darker intensity than the same fragment in other Sst I digestions, was a doublet containing a fragment relating to the ~0.9 kb Xba I/Eco RI product. That is, the Eco RI restriction site currently mapped ~1.0 kb from the left arm was possibly 0.9 kb from the cloning junction and represented in the present Sst I/Eco RI digest by a ~0.86 kb fragment. At this point, only the ~4.9 kb and ~2.9 kb Sst I fragments had been mapped within λOHNZ1, however the Kpn I/Sst I digest provided evidence for the positioning of one of the ~2.65 kb Sst I fragments.

As expected from the map, the ~4.9 kb Sst I fragment was not cleaved by Kpn I (Figure 24). Kpn I cleavage of the ~2.9 kb Sst I fragment was predicted to yield fragments of ~2.55, 0.3 and 0.1 kb. A ~2.5 kb fragment was visible below the ~2.65 kb Sst I fragment. For a ~2.65 kb Sst I fragment to be cleaved by Kpn I, it would be located within the central region of the clone (Figure 25). The unique ~1.55 kb and ~1.05 kb Kpn I/Sst I fragments implied that the ~2.65 kb Sst I fragment was immediately adjacent to the ~2.9 kb Sst I fragment. The presence of the ~1.55 kb Kpn I/Sst I fragment allowed the order of the ~0.2 kb and ~1.65 kb Kpn I fragments to be defined. The ~0.2 kb fragment was positioned between the ~8.8 kb and ~1.65 kb Kpn I fragments on the map (Figure 25). This placed the ~1.65 kb Kpn I fragment such that Sst I cleavage of this fragment would yield a ~1.55 kb Sst I/Kpn I fragment. The other ~2.65 kb Sst I fragment which was not cleaved by Kpn I was concluded to lie further 5' in the clone along with the ~2.2, 0.86 and ~0.74 kb Sst I fragments. The ~1.45 kb Kpn I fragment provided further evidence that it is this fragment which resulted from Kpn I

digestion in the left arm. As observed in the earlier Kpn I/Not I digest, the ~1.45 kb Kpn I fragment was co-migrating with a ~1.45 kb Sst I/Kpn I fragment.

Within the Sst I/Eco RI digest, a ~2.65 kb fragment positioned in the centre of the clone accounted for the ~1.95 kb and ~0.5 kb Sst I/Eco RI fragments (Figure 24). Furthermore, the ~1.95 kb fragment would originate from Sst I and Eco RI cleavage, establishing that the ~2.0 kb Eco RI fragment was cleaved by Sst I and therefore absent from the double digest. The ~2.2 kb Sst I fragment was cleaved by Eco RI indicating that this fragment was not next to the ~2.65 kb Sst I fragment (Figure 24). Similarly, the other ~2.65 kb Sst I fragment could not be located in this region of the clone as this would not have resulted in Eco RI cleavage of this fragment. Thus either the ~0.86 or ~0.72 kb Sst I fragment lies adjacent to the central ~2.65 kb Sst I fragment. To establish which fragment lies beside the ~2.65 kb Sst I fragment in the clone, it would be necessary to compare an Sst I digest with a Sma I/Sst I profile. Sma I should cleave one but not both of these fragments when a recognition site is adjacent to the ~2.65 kb Sst I fragment. Establishing which fragment was cleaved by Sma I would define which fragment is adjacent to the ~2.65 kb Sst I. The source of the other Sst I/Eco RI fragments (Table 12) remains to be defined.

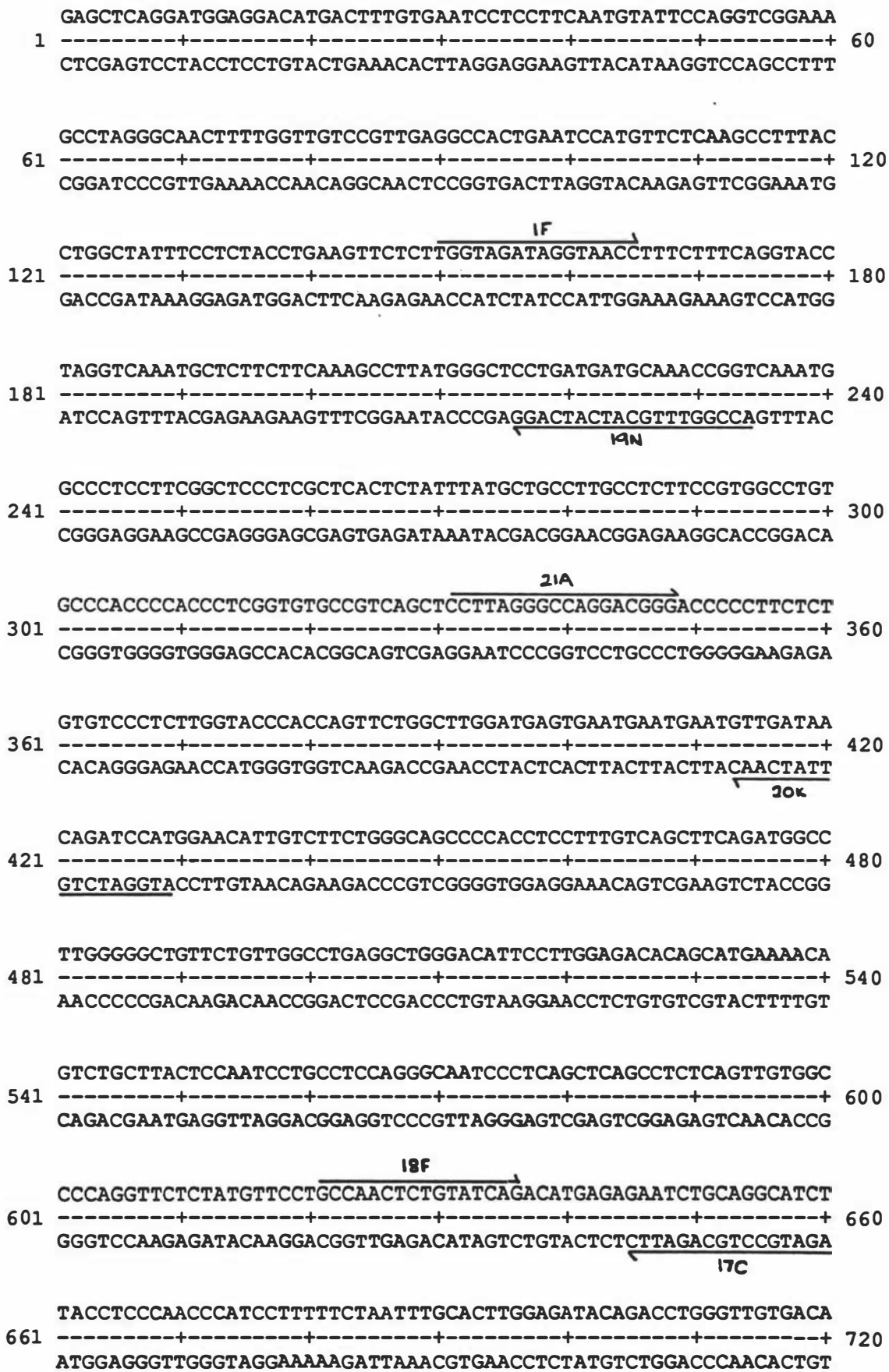
Double digestion with Sst I and Xba I produced a single ~2.2 kb fragment (Figure 16A). As both Sst I and Xba I single digestions contained a ~2.2 kb fragment, the origin of the 2.2 kb fragment within the Sst I/Xba I double digestion was unknown. The ~2.2 kb Xba I fragment had been mapped adjacent to the ~3.2 kb Xba I fragment (Figure 20). One of the Sst I restriction sites responsible for the mapped ~2.65 kb Sst I fragment was within the ~2.2 kb Xba I fragment. Therefore, it was concluded that the Sst I/Xba I digest contained the Sst I ~2.2 kb fragment (Figure 16A; Table 8).

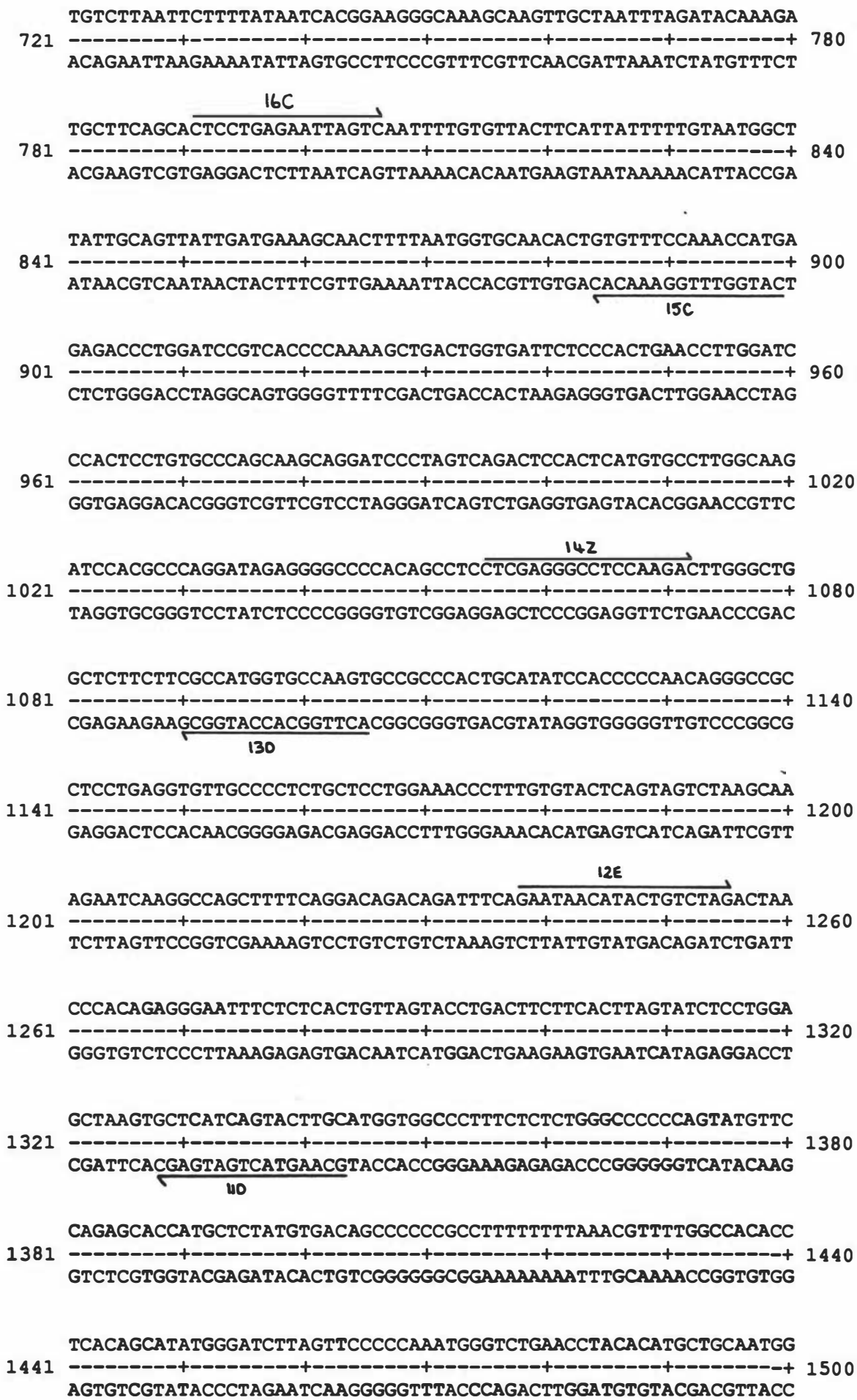
Xho I cleaved within the ~7.44 kb Kpn I fragment producing the ~4.9 kb Xho I fragment and ~3.4 kb and ~0.72 kb Xho I/Kpn I fragments. The ~3.4 kb fragment was 5' to the ~1.45 kb fragment which resulted from Kpn I digestion in the left arm. A value of ~3.24 kb was predicted from the map for this fragment (Figure 25). The ~0.16 kb difference in these values may have resulted from experimental error or may have indicated that the internal Kpn I site was not exactly 18.56 kb from the end of the left arm as suggested in figure 9. This might also have explained the inconsistency of a Kpn I fragment (1.45 kb cf. 1.51 kb). The ~0.89 kb partial digestion product implied that a ~0.17 kb Kpn I fragment was in the centre of the clone, adjacent to the ~0.72 kb Xho I/Kpn I fragment.

As Xho I cleaved only twice in λOHNZ1, the Xho I/Sst I digest was dominated by Sst I fragments. Xho I digestion of the ~4.9 kb Sst I fragment produced the ~3.15 kb and ~1.85 kb Sst I/Xho I fragments (Figure 25). The rest of the ~4.8 kb fragment Xho I was contained within the ~1.65 kb Sst I/Xho I fragment while the ~1.05 kb Sst I/Xho I fragment represented the remainder of the ~2.9 kb Sst I fragment (Figure 25). A ~0.42 kb fragment of unknown origin was also present in this digest along with a ~4.9 kb partial fragment.

The triple digests of Xho I/Kpn I/Eco RI and Xho I/Kpn I/Sst I, confirmed the position of the mapped restriction sites in λOHNZ1 for these enzymes. The restriction map did not explain the origin of the ~0.45 kb fragment in the Xho I/Kpn I/Sst I digest. This additional fragment of unknown source may have been in part related to some of the Sst I restriction sites being undefined. Figure 25 summarises the digestion profiles seen on figure 24.

Appendix 4: Nucleotide sequence of 2.8 kb Sst I fragment isolated from the bovine lactoferrin genomic clone λOHNZ1

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16C
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781 ---------+---------+---------+---------+---------+---------+ 840
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841 ---------+---------+---------+---------+---------+---------+ 900
ATAACGTCAATAACTACTTTCGTTGAAAATTACCACGTTGTGACACAAAGGTTTGGTACT
15C
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901 ---------+---------+---------+---------+---------+---------+ 960
CTCTGGGACCTAGGCAGTGGGGTTTTCGACTGACCACTAAGAGGGTGACTTGGAACCTAG
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961 ---------+---------+---------+---------+---------+---------+ 1020
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14Z
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1021 ---------+---------+---------+---------+---------+---------+ 1080
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GCTCTTCTTCGCCATGGTGCCAAGTGCCGCCCACTGCATATCCACCCCCAACAGGGCCGC
1081 ---------+---------+---------+---------+---------+---------+ 1140
CGAGAAGAAGCGGTACCACGGTTCACGGCGGGTGACGTATAGGTGGGGGTTGTCCCGGCG
13O
CTCCTGAGGTGTTGCCCCTCTGCTCCTGGAAACCCTTTGTGTACTCAGTAGTCTAAGCAA
1141 ---------+---------+---------+---------+---------+---------+ 1200
GAGGACTCCACAACGGGGAGACGAGGACCTTTGGGAAACACATGAGTCATCAGATTCGTT
12E
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1201 ---------+---------+---------+---------+---------+---------+ 1260
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1261 ---------+---------+---------+---------+---------+---------+ 1320
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GCTAAGTGCTCATCAGTACTTGCATGGTGGCCCTTTCTCTCTGGGCCCCCCAGTATGTTC
1321 ---------+---------+---------+---------+---------+---------+ 1380
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11D
CAGAGCACCATGCTCTATGTGACAGCCCCCCGCCTTTTTTTTAAACGTTTTGGCCACACC
1381 ---------+---------+---------+---------+---------+---------+ 1440
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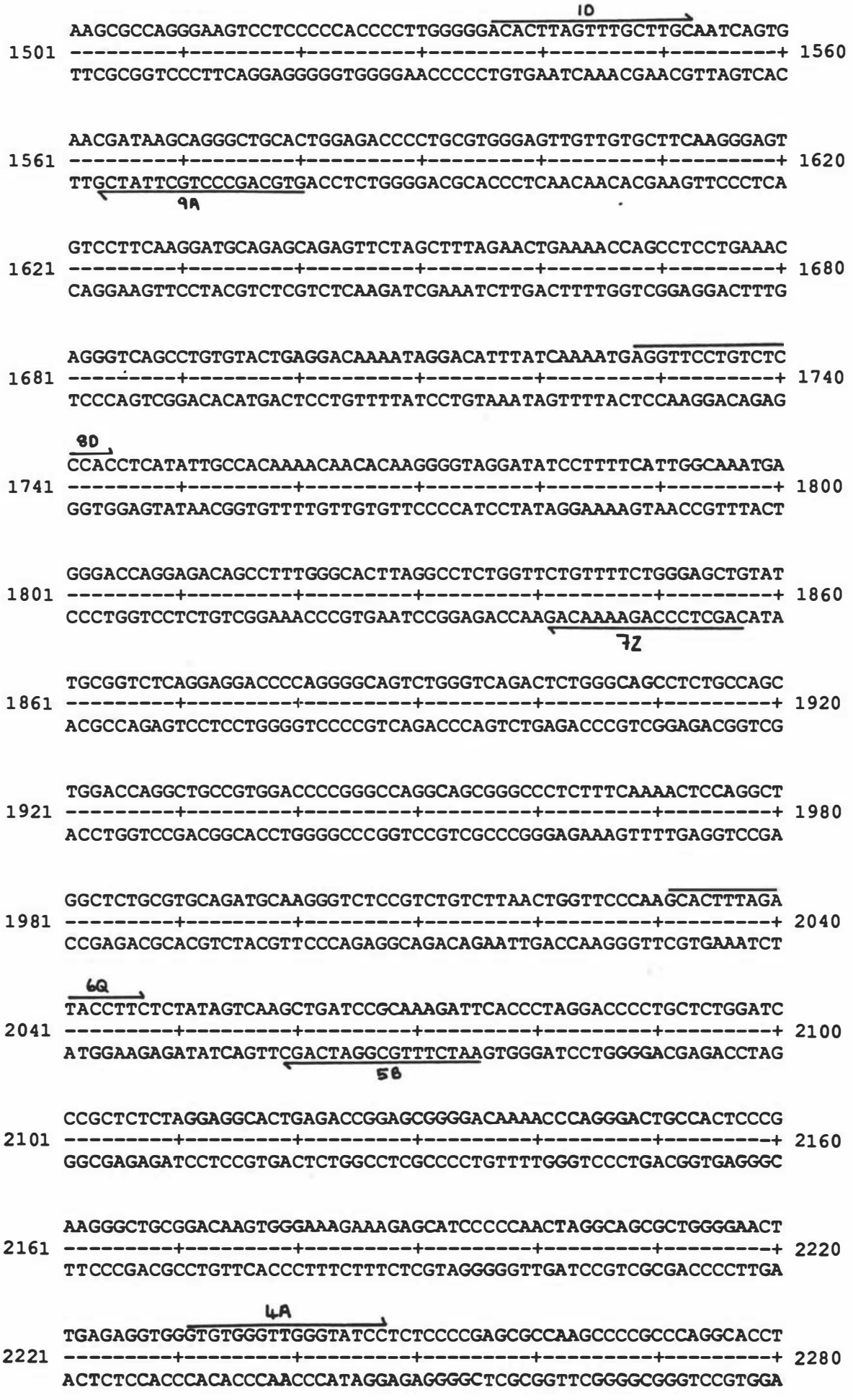
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9A
GTCCTTCAAGGATGCAGAGCAGAGTTCTAGCTTTAGAACTGAAAACCAGCCTCCTGAAAC
1621 ---------+---------+---------+---------+---------+---------+ 1680
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1681 ---------+---------+---------+---------+---------+---------+ 1740
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8D
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1741 ---------+---------+---------+---------+---------+---------+ 1800
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GGGACCAGGAGACAGCCTTTGGGCACTTAGGCCTCTGGTTCTGTTTTCTGGGAGCTGTAT
1801 ---------+---------+---------+---------+---------+---------+ 1860
CCCTGGTCCTCTGTCGGAAACCCGTGAATCCGGAGACCAAGACAAAAGACCCTCGACATA
7Z
TGCGGTCTCAGGAGGACCCCAGGGGCAGTCTGGGTCAGACTCTGGGCAGCCTCTGCCAGC
1861 ---------+---------+---------+---------+---------+---------+ 1920
ACGCCAGAGTCCTCCTGGGGTCCCCGTCAGACCCAGTCTGAGACCCGTCGGAGACGGTCG
TGGACCAGGCTGCCGTGGACCCCGGGCCAGGCAGCGGGCCCTCTTTCAAAACTCCAGGCT
1921 ---------+---------+---------+---------+---------+---------+ 1980
ACCTGGTCCGACGGCACCTGGGGCCCGGTCCGTCGCCCGGGAGAAAGTTTTGAGGTCCGA
GGCTCTGCGTGCAGATGCAAGGGTCTCCGTCTGTCTTAACTGGTTCCCAAGCACTTTAGA
1981 ---------+---------+---------+---------+---------+---------+ 2040
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6Q
TACCTTCTCTATAGTCAAGCTGATCCGCAAAGATTCACCCTAGGACCCCTGCTCTGGATC
2041 ---------+---------+---------+---------+---------+---------+ 2100
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5B
CCGCTCTCTAGGAGGCACTGAGACCGGAGCGGGGACAAAACCCAGGGACTGCCACTCCCG
2101 ---------+---------+---------+---------+---------+---------+ 2160
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2161 ---------+---------+---------+---------+---------+---------+ 2220
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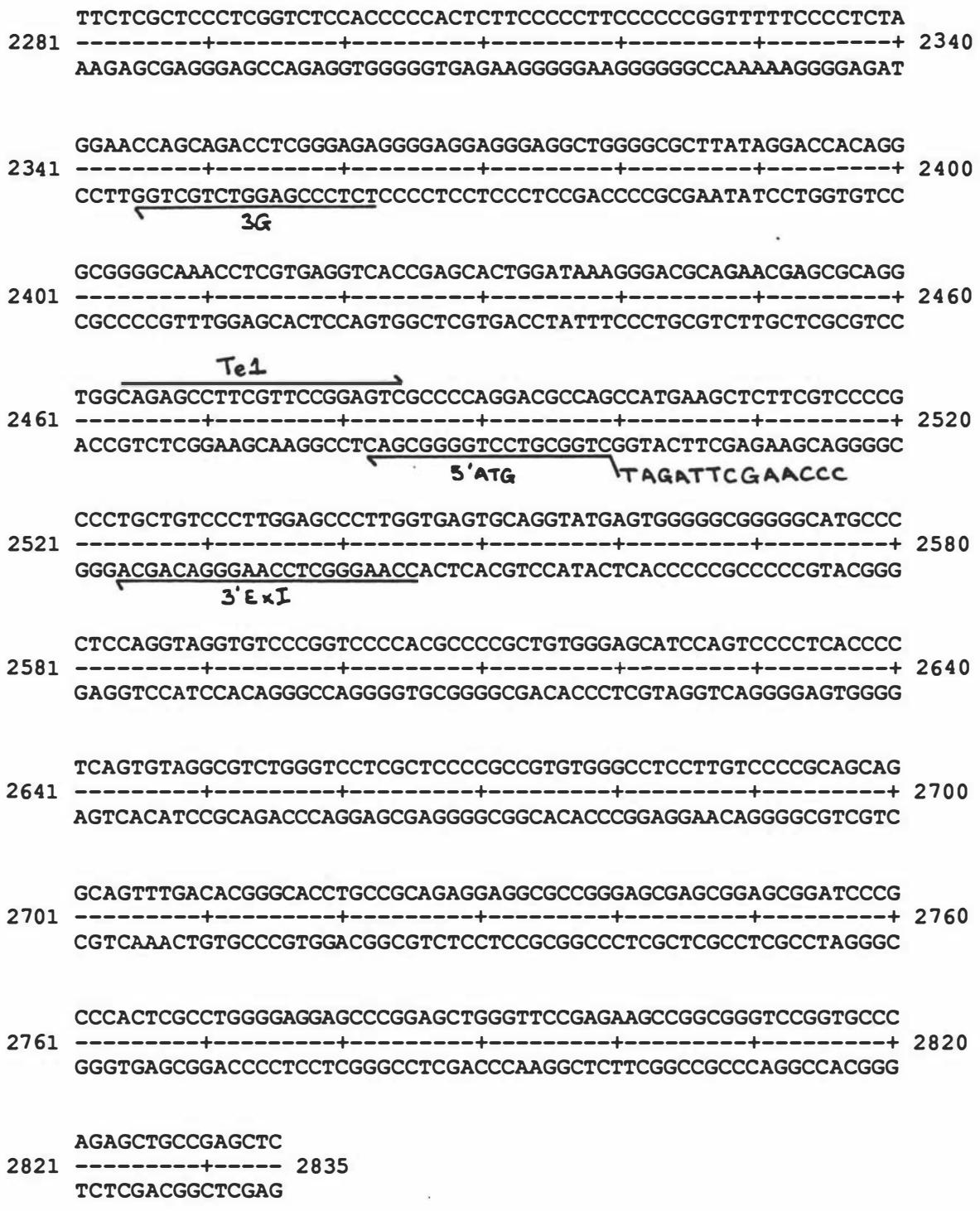
4A
TGAGAGGTGGGTGTGGGTTGGGTATCCTCTCCCCGAGCGCCAAGCCCCGCCCAGGCACCT
2221 ---------+---------+---------+---------+---------+---------+ 2280
ACTCTCCACCCACACCCAACCCATAGGAGAGGGGCTCGCGGTTCGGGGCGGGTCCGTGGA


TTCTCGCTCCCTCGGTCTCCACCCCCACTCTTCCCCCTTCCCCCCGGTTTTTCCCCTCTA
2281 ---------+---------+---------+---------+---------+---------+ 2340
AAGAGCGAGGGAGCCAGAGGTGGGGGTGAGAAGGGGGAAGGGGGGCCAAAAAGGGGAGAT
GGAACCAGCAGACCTCGGGAGAGGGGAGGAGGGAGGCTGGGGCGCTTATAGGACCACAGG
2341 ---------+---------+---------+---------+---------+---------+ 2400
CCTTGGTCGTCTGGAGCCCTCTCCCCTCCTCCCTCCGACCCCGCGAATATCCTGGTGTCC
3G
GCGGGGCAAACCTCGTGAGGTCACCGAGCACTGGATAAAGGGACGCAGAACGAGCGCAGG
2401 ---------+---------+---------+---------+---------+---------+ 2460
CGCCCCGTTTGGAGCACTCCAGTGGCTCGTGACCTATTTCCCTGCGTCTTGCTCGCGTCC
Te1
TGGCAGAGCCTTCGTTCCGGAGTCGCCCCAGGACGCCAGCCATGAAGCTCTTCGTCCCCG
2461 ---------+---------+---------+---------+---------+---------+ 2520
ACCGTCTCGGAAGCAAGGCCTCAGCGGGGTCCTGCGGTCGGTACTTCGAGAAGCAGGGGC
5'ATG
TAGATTCGAACCC
CCCTGCTGTCCCTTGGAGCCCTTGGTGAGTGCAGGTATGAGTGGGGGCGGGGGCATGCCC
2521 ---------+---------+---------+---------+---------+---------+ 2580
GGGACGACAGGGAACCTCGGGAACCACTCACGTCCATACTCACCCCCGCCCCCGTACGGG
3'ExI
CTCCAGGTAGGTGTCCCGGTCCCCACGCCCCGCTGTGGGAGCATCCAGTCCCCTCACCCC
2581 ---------+---------+---------+---------+---------+---------+ 2640
GAGGTCCATCCACAGGGCCAGGGGTGCGGGGCGACACCCTCGTAGGTCAGGGGAGTGGGG
TCAGTGTAGGCGTCTGGGTCCTCGCTCCCCGCCGTGTGGGCCTCCTTGTCCCCGCAGCAG
2641 ---------+---------+---------+---------+---------+---------+ 2700
AGTCACATCCGCAGACCCAGGAGCGAGGGGCGGCACACCCGGAGGAACAGGGGCGTCGTC
GCAGTTTGACACGGGCACCTGCCGCAGAGGAGGCGCCGGGAGCGAGCGGAGCGGATCCCG
2701 ---------+---------+---------+---------+---------+---------+ 2760
CGTCAAACTGTGCCCGTGGACGGCGTCTCCTCCGCGGCCCTCGCTCGCCTCGCCTAGGGC
CCCACTCGCCTGGGGAGGAGCCCGGAGCTGGGTTCCGAGAAGCCGGCGGGTCCGGTGCCC
2761 ---------+---------+---------+---------+---------+---------+ 2820
GGGTGAGCGGACCCCTCCTCGGGCCTCGACCCAAGGCTCTTCGGCCGCCCAGGCCACGGG
AGAGCTGCCGAGCTC
2821 ---------+----- 2835
TCTCGACGGCTCGAG

Appendix 5: Nucleotide alignment of bovine lactoferrin promoter and exon I sequences.

Comparison of sequence deduced by this study (nt 1453-2542) and by Seyfert *et al.*, 1994 (nt 1-1088). Percentage similarity of sequences was determined as 99.283% using the Genetics Computer Group (GCG) programmes. The translational start codon is underlined.

```
   1 GATCTTAGTTCCCCCAAATGGGTCTGAACCTACACATGCTGCAATGGAAG 50
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1453 GATCTTAGTTCCCCCAAATGGGTCTGAACCTACACATGCTGCAATGGAAG 1502

  51 CGCCAGGGAAGTCCTCCCCCACCCCTTGGGGGACACTTAGTTTGCTTGCA 100
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1503 CGCCAGGGAAGTCCTCCCCCACCCCTTGGGGGACACTTAGTTTGCTTGCA 1552

 101 ATCAGTGAACGATAAGCAGGGCTGCACTGGAGACCCCTGCGTGGGAGTTG 150
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1553 ATCAGTGAACGATAAGCAGGGCTGCACTGGAGACCCCTGCGTGGGAGTTG 1602

 151 TTGTGCTTCAAGGGAGTGT.CTTCAAGGATGCAGAGCAGAGTTCTAGC.T 198
     |||||||||||||||||| |||||||||||||||||||||||||||| |
1603 TTGTGCTTCAAGGGAGTGTCCTTCAAGGATGCAGAGCAGAGTTCTAGCTT 1652

 199 TAGAACTGAAAACCAGCCTCCTGAAACAGGGTCAGCCTGTGTACTGAGGA 248
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1653 TAGAACTGAAAACCAGCCTCCTGAAACAGGGTCAGCCTGTGTACTGAGGA 1702

 249 CAAAATAGGACATTTATCAAAATGAGGTTCCTGTCTCCCACCTCATATTG 298
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1703 CAAAATAGGACATTTATCAAAATGAGGTTCCTGTCTCCCACCTCATATTG 1752

 299 CCACAAAACAACACAAGGGGTAGGATATCCTTTTCATTGGCAAATGAGGG 348
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1753 CCACAAAACAACACAAGGGGTAGGATATCCTTTTCATTGGCAAATGAGGG 1802

 349 ACCAGGAGACAGCCTTTGGGCACTTAGGCCTCTGGTTCTGTTTTCTGGGA 398
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1803 ACCAGGAGACAGCCTTTGGGCACTTAGGCCTCTGGTTCTGTTTTCTGGGA 1852

 399 GCTGTATTTCGGTCTCAGGAGGACTCCAGGGGCAGTCTGGGTCAGACTCT 448
     |||||||| |||||||||||||| |||||||||||||||||||||||||
1853 GCTGTATTGCGGTCTCAGGAGGACCCCAGGGGCAGTCTGGGTCAGACTCT 1902

 449 GGGCAGCCTCTGCCAGCTGGACCAGGCTGCCGTGGACCCCGGGCCAGGCA 498
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1903 GGGCAGCCTCTGCCAGCTGGACCAGGCTGCCGTGGACCCCGGGCCAGGCA 1952

 499 GCGGGCCCTCTTTCAAAACTCCAGGCTGGCTCTGCGTGCAGATGCAAGGG 548
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1953 GCGGGCCCTCTTTCAAAACTCCAGGCTGGCTCTGCGTGCAGATGCAAGGG 2002
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```
 549 TCTCCGTCTGTCTTAACTGGTTCCCAAGCACTTTAGATACCTTCTCTATA 598
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2003 TCTCCGTCTGTCTTAACTGGTTCCCAAGCACTTTAGATACCTTCTCTATA 2052

 599 GTCAAGCTGATCCGCAAAGATTCACCCTAGGACCCCTGCTCTGGATCCCG 648
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2053 GTCAAGCTGATCCGCAAAGATTCACCCTAGGACCCCTGCTCTGGATCCCG 2102

 649 CTCTCTAGGAGGCACTGAGACCGGAGCGGGGACAAAACCCAGGGACTGCC 698
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2103 CTCTCTAGGAGGCACTGAGACCGGAGCGGGGACAAAACCCAGGGACTGCC 2152

 699 ACTCCCGAAGGGCTGCGGACAAGTGGGAAAGAAAGAGC..ACCCCCCAAC 746
     ||||||||||  |||||||||||||||||||||||||  | ||||||||
2153 ACTCCCGAAG..CTGCGGACAAGTGGGAAAGAAAGAGCATAACCCCCAAC 2200

 747 TAGGCAGCGCTGGGGAACTTGAGAGGTGGGTGTGGGTTGGGTATCCTCTC 796
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2201 TAGGCAGCGCTGGGGAACTTGAGAGGTGGGTGTGGGTTGGGTATCCTCTC 2250

 797 CCCGAGCGCCAAGCCCCGCCCGGGCACCTTTCTCGCTCCCTCGGTCTCCA 846
     |||||||||||||||||||| ||||||||||||||||||||||||||||
2251 CCCGAGCGCCAAGCCCCGCCCAGGCACCTTTCTCGCTCCCTCGGTCTCCA 2300

 847 CCCCCGCTCTTCCCCCTTCCCCCCGGTTTTCCCCCTCTAGGAACCAGCAG 896
     |||||  ||||||||||||||||||||||  ||||||||||||||||||
2301 CCCCCACTCTTCCCCCTTCCCCCCGGTTTTTCCCCTCTAGGAACCAGCAG 2350

 897 ACCTCGGGAGAGGGGAGGAGGGAGGCTGGGGCGCTTATAGGACCACAGGG 946
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2351 ACCTCGGGAGAGGGGAGGAGGGAGGCTGGGGCGCTTATAGGACCACAGGG 2400

 947 CGGGGCAAACCTCGTGAGGTCACCGAGCACTGGCTAAAGGGACGCAGAAC 996
     |||||||||||||||||||||||||||||||  ||||||||||||||||
2401 CGGGGCAAACCTCGTGAGGTCACCGAGCACTGGATAAAGGGACGCAGAAC 2450

 997 GAGCGCAGGTGGCAGAGCCTTCGTTCCGGAGTCGCCCCAGGACGCCAGCC 1046
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2451 GAGCGCAGGTGGCAGAGCCTTCGTTCCGGAGTCGCCCCAGGACGCCAGCC 2500

1047 ATGAAGCTCTTCGTCCCCGCCCTGCTGTCCCTCGGAGCCCTT          1088
     |||||||||||||||||||||||||||||||  |||||||||
2501 ATGAAGCTCTTCGTCCCCGCCCTGCTGTCCCTTGGAGCCCTT          2542
```